Docket No.: 595792000420
(PATENT)

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

In re Patent Application of:
Allen G. GOOD et al.

Application No.: 10/756,213 | Confirmation No.: 1748

Filed: January 12, 2004 | Art Unit: 1638

For: PLANTS WITH ENHANCED LEVELS OF NITROGEN UTILIZATION PROTEINS IN THEIR ROOT EPIDERMIS AND USES THEREOF | Examiner: D. Kruse

DECLARATION OF JEAN C. KRIDL UNDER 37 C.F.R. §1.132

MS RCE
Commissioner for Patents
P.O. Box 1450
Alexandria, VA 22313-1450

Dear Sir:

1. I, Jean C. Kridl, do hereby state and declare that all statements made herein of my own knowledge are true and that all statements made on information and belief are believed to be true; and further that these statements were made with the knowledge that willful false statements and the like so made are punishable by fine or imprisonment, or both, under §1001 of Title 18 of the United States Code, and that such willful false statements may jeopardize the validity of the application or any patent issuing thereon.

2. I, Jean C. Kridl, am a U.S. citizen and am currently employed as a senior scientist in Arcadia Biosciences, Inc., the licensee of the present application. As an employee of Arcadia Biosciences, Inc., I receive remuneration and have certain stock options in Arcadia Biosciences, Inc.; therefore I have an interest in the issuance of the above referenced patent application. I have worked in the field of plant biochemistry and molecular biology for 30 years. I have a Ph.D. degree in Biochemistry from University of California, Davis; and a

B.A. degree in Biochemistry and Molecular Biology from the University of California, Santa Barbara. A copy of my *Curriculum Vitae* is attached as Exhibit 1.

3. I have read U.S. Patent Application No. 10/756,213, the Office Action mailed May 25, 2007, the prior Office Action mailed August 24, 2006, and the references cited therein. I understand that the Examiner in the May 25, 2007 Office Action is of the view that claims directed to transgenic plants transformed with barley AlaAT are obvious in view of Coruzzi *et al.* (U.S. Patent No. 5,955,651) and Muench *et al.* (1994; *Plant Mol. Biol.* 24:417-427) and Suzuki et al. (January 1993, *Plant Mol. Biol.* 21: 109-119).

4. In these Office Actions, the Examiner states that "those of ordinary skill at the time of Applicant's invention would have been motivated to introduce a transgene encoding alanine aminotransferase in plants." As I understand it based on the arguments presented in both Office Actions, the Examiner's argument that the teachings of Coruzzi, Muench, and Suzuki render the claims obvious is based on the following logic:

> (A) Coruzzi teaches that the steady flow of nitrogen from ammonia to asparagine in this [nitrogen assimilatory] pathway depends on the recycling of glutamate and alpha-ketoglutarate (Column 2, 2nd paragraph).
>
> (B) The fact that alanine aminotransferase recycles glutamate and alpha ketoglutarate would have been common knowledge to those of ordinary skill in the art.
>
> (C) Muench teaches a nucleic acid encoding a barley AlaAT-2 polypeptide.
>
> (D) Suzuki teaches a root-epidermis-specific promoter.
>
> (E) Given the success of Coruzzi *et al.* in expressing amino acid synthetase enzyme encoding nucleic acids in transgenic plants and producing transgenic plants with enhanced nitrogen assimilation, one of ordinary skill would have had a reasonable expectation of success in modifying their teachings using the nucleic acid taught by Muench *et al.* or the promoter of Suzuki *et al.*

5. Regarding (A), the Examiner's statement is factually correct but incomplete. Coruzzi does state that "[t]he steady flow of nitrogen from ammonia to asparagine in this pathway depends upon the recycling of glutamate and alpha-ketoglutarate and aspartate." The

Examiner fails to point out that the sentence at Column 2, 2nd paragraph of Coruzzi continues and states the recycling of glutamate and alpha-ketoglutarate and aspartate is ***"catalyzed by glutamine 2:oxoglutarate aminotransferase (GOGAT; E.C.) and aspartate aminotransferase (AspAT; E.C.), respectively (see FIG. 1). Thus, GS, AS, AspAT and GOGAT comprise the key enzymes of the main nitrogen assimilatory pathway of higher plants.***" Coruzzi clearly does not state that such recycling of glutamate and alpha-ketoglutarate and aspartate would be facilitated by alanine aminotransferase much less any other enzyme that could participate in such recycling other than GOGAT and AspAT. Any enzyme that catalyzes the conversion of glutamate to alpha-ketoglutarate or the reverse reaction will have an unknown impact on the entire nitrogen assimilation and utilization pathway as shown by Coruzzi in Fig. 1. Any such enzyme could essentially "short circuit" the nitrogen assimilation pathway by competing for substrates with key enzymes in the nitrogen assimilation pathway, AspAT, GS, and GOGAT. One of skill in the art could not have predicted whether such a short circuiting would have negatively or positively affected the cycle.

6. Regarding (B), the examiner's statement that "alanine aminotransferase recycles glutamate and alpha-ketoglutarate would have been common knowledge to those of ordinary skill in the art" greatly oversimplifies the complexity of this enzyme. AlaAT is a freely reversible enzyme that synthesizes the amino acid alanine from pyruvate by transferring an amino group from glutamate. In the reverse reaction, AlaAT transfers the amino group from alanine to alpha-ketoglutarate to regenerate glutamate. As of the priority date, one of skill in the art would not have known whether AlaAT when expressed transgenically would function in the forward direction or in the reverse direction.

7. Many aminotransferases are documented in the literature and they are known to use a variety of substrates, including alanine, *in vitro*; however, at least as of the filing date of the application, the substrates that these enzymes use *in vivo* was not entirely clear. Since the substrates that these enzymes will use *in vivo* were not entirely clear, it would have been impossible to predict whether over expression of the enzyme would have a positive or negative effect on the resulting transgenic plant. The attached article (Exhibit 2) by Gloria

Coruzzi published even after the priority date of this application, The *Arabidopsis* Book, American Society of Plant Biologists, September 30, 2003, supports this position. At page 1, paragraph 1, Coruzzi states:

> "As the mechanisms controlling intra- and intercellular transport of inorganic and organic nitrogen in plants are still under investigation, it is impossible to predict the *in vivo* function of nitrogen assimilatory enzymes localized in distinct cells or subcellular compartments based on *in vitro* biochemistry."

8. I disagree with the Examiner's statement in (E) that "Given the success of Coruzzi *et al.* in expressing amino acid synthetase enzyme encoding nucleic acids in transgenic plants and producing transgenic plants with enhanced nitrogen assimilation, one of ordinary skill would have had a reasonable expectation of success in modifying their teachings using the nucleic acid taught by Muench *et al.* or the promoter of Suzuki *et al.*" While Coruzzi demonstrates that expressing certain enzymes implicated in amino acid biosynthesis produces transgenic plants with enhanced nitrogen assimilation, there is nothing to suggest that over-expression of any enzyme involved in amino acid biosynthesis in plants would lead to increased nitrogen assimilation and biomass. In fact, in a 1996 publication showed that over-production of lysine in *Arabidopsis* produced plants with abnormal phenotypes (Tzchori et al., 1996 *Plant Mol. Biol.* 32 (4): 727-34) (Exhibit 3). Over expression of cystathionine gamma synthase, a key enzyme in the biosynthetic pathway to methionine (Met), produced *Arabidopsis* plants with a normal phenotype and greatly increased levels of Met (Kim *et al.*, 2002 *Plant Physiol.* 128: 95-107) (Exhibit 4). Plants over-expressing Met are not known to have nitrogen use efficiency. Tryptophan accumulation is detected in transgenic rice seeds by over-expression of a feedback insensitive alpha subunit of rice antranilate synthase and the plants are reported to be reduced in spikelet fertility, yield and seed germination ability compared to wild type (Wakasa *et al.*, 2006 *J. Exp. Bot.* 57:3069-3078) (Exhibit 5). There is no report of nitrogen use efficiency in the rice. Thus, one of skill in the art could not predict whether or not over expression of any enzyme involved in amino acid biosynthesis would have a positive or negative impact on the plant much less whether it would lead to increased nitrogen assimilation.

9. Even the inventors did not predict that transgenic expression of AlaAT in plants would result in enhanced nitrogen utilization efficiency before their invention. It is my understanding that the initial transgenic plants were produced by Good *et al.* in an attempt to use alanine as a compatible solute to confer drought and/or salt tolerance to *Brassica* plants. The promoter chosen for this work was the btg26 promoter which is inducible under conditions of osmotic stress, salt and abscisic acid treatment. The gene of interest to confer synthesis of alanine was the AlaAT gene from barley. In the process of growing the transgenic plants in pots for drought and salt tolerance studies, the plants were mistakenly treated with water alone rather than the normal fertilization mix containing nitrogen, phosphorus and potassium supplementation. It was noted that the transgenic plants had increased growth under these nitrogen-deficient conditions compared to control plants. Thus, it is my understanding that the intent of Good *et al.*'s experiment was to study salt and drought tolerance and in fact they made a serendipitous discovery about nitrogen utilization.

10. Unlike aspartate and asparagine biosynthesis, alanine biosynthesis is not commonly regarded as a core part of the nitrogen cycle. Biochemistry textbooks, both old and new, do not include AlaAT when discussing the nitrogen cycle and in fact it is rare to see reference to AlaAT in textbooks in any context besides liver function in humans. Coruzzi describes the nitrogen cycle extensively in U.S. Patent No. 5,955,651 and does not mention alanine aminotransferase or alanine in any context. Glutamine, aspartate and asparagine are well documented as nitrogen transport amino acids, but alanine is not documented in this context. Four AlaAT genes have been identified in *Arabidopsis* and information is publically available on the TAIR website (www.arabidopsis.org) from numerous transcription profiling experiments (under the Genevestigator Response Viewer). For three of the four AlaAT genes where data is available, the genes are not significantly affected under conditions of nitrogen starvation and resupply supporting the notion that these genes are not normally involved in nitrogen assimilation.

11. Alanine in plants is known to increase under hypoxic and anaerobic stress conditions. An anaerobically-induced isoform of AlaAT in barley, AlaAT-2, was shown to increase under different levels of hypoxia with activity increasing with lower levels of oxygen. This

induction was not affected by the levels of nitrate available to the plants in the media, thus the alanine aminotransferase induced during hypoxia was not known to be affected by nitrogen. *See* Good and Crosby (1989) *Plant Physiol.* 90:1305-1309 (Exhibit 6). The gene encoding this anaerobically induced isoform of AlaAT was cloned from barley and used in the experiments described in the present application.

12. Even if one of skill in the art were motivated to select an amino acid biosynthetic enzyme to over express in an attempt to enhance nitrogen metabolism, there are many such biosynthetic enzymes from which to choose. Thus, AlaAT is only one enzyme among a large number, none of which Corruzi mentions and therefore Coruzzi provides no guidance as to which to test in order to determine which would actually enhance nitrogen assimilation. Examples of aminotransferases and enzymes involved in amino acid biosynthesis include without limitation, alanine aminotransferase, glycine aminotransferase, 2-aminoadipate aminotransferase, 4-aminobutyrate aminotransferase, succinyldiaminopimelate aminotransferase, tryptophan aminotransferase, branched chain amino acid aminotransferease, phophoserine aminotransferase, acetylornithine aminotransferase, ornithine-oxo-acid aminotransferase, glucosamine-fructose-6-phosphate aminotransferase, glutamate-1-semialdehyde aminotransferase, histidinol-phosphate aminotransferease, aparatate aminotransfersase, tyrosine aminotransferase, beta-alanine-pyruvate aminotransferase, adenosylmethionine-8-7-oxononanoate aminotransferase, anthranilate synthase, chorismate mutase, prephenate aminotransferase, tryptophan synthase, dihydrodipicolinate synthase, cystathionine gamma synthase, and threonine synthase.

13. In addition, alanine has been shown to feedback inhibit the GS enzyme in bacteria (see, *e.g.*, S.-H. Liaw et al. *PNAS* (1993) 90:4996-5000) (Exhibit 7). Alanine has also been shown to inhibit one GS from plant at least slightly (*see*, T. D. O'Neal and K. W. Joy *Plant Physiol* (1975) 55:968-974) (Exhibit 8). Thus, even if one were motivated to select an amino acid biosynthetic enzyme in an attempt to increase nitrogen assimilation, one of skill in the art would be motivated to select a synthetic enzyme for an amino acid that has not been shown to inhibit GS. One of skill in the art would have expected that over expression of alanine

would have affected GS activity which would interfere with nitrogen assimilation and utilization and therefore would not have chosen to over-express AlaAT in plants.

14. Given the knowledge of the respective genes at the time of the invention, it is my opinion that the inventors' over-expression of the barley AlaAT gene was not obvious as a means to enhance nitrogen utilization efficiency (NUE) in plants. The gene had not been shown to be regulated by nitrogen and there was no evidence that endogenous AlaAT play a role in nitrogen assimilation in plants until this application. Therefore, it would not have been obvious at the time of the invention for one skilled in the art to expect that over-expression of barley AlaAT would enhance NUE. Because there are numerous AlaAT genes (two to six plant isozymes) with distinct subcellular localizations (cytosol, mitochondria, peroxisomes), it would not be reasonable to assume that over-expression of any AlaAT gene would have the same effect of increasing NUE.

15. In my scientific opinion, the observation by the inventors that over-expression of barley AlaAT results in enhanced NUE and increased biomass is a surprising finding that could not have been predicted by one of skill in the art.

10/31/07 Jean C. Kridl

Date Jean C. Kridl

Attachments

Exhibit 1: Jean C. Kridl's Curriculum Vitae
Exhibit 2: Gloria M. Coruzzi, *The Arabidopsis Book*
Exhibit 3: Tzchori et al., 1996 *Plant Mol. Biol.* 32 (4): 727-34
Exhibit 4: Kim *et al.*, 2002 *Plant Physiol.* 128: 95-107
Exhibit 5: Wakasa *et al.*, 2006 *J. Exp. Bot.* 57:3069-3078
Exhibit 6: Good and Crosby (1989) *Plant Physiol.* 90:1305-1309
Exhibit 7: S.-H. Liaw et al. *PNAS* (1993) 90:4996-5000
Exhibit 8: T. D. O'Neal and K. W. Joy *Plant Physiol* (1975) 55:968-974

Exhibit 1

Jean C. Kridl

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SUMMARY

A Ph.D. plant molecular biologist with 22 years experience in the agricultural biotechnology industry. Proven skills in technology management and multidisciplinary, multi-location project management. Published and patented record of scientific achievement in discovery research. Respected for science communication skills and mentoring young scientists.

PROFESSIONAL EXPERIENCE

Arcadia Biosciences, Davis, CA **2004-present**

Senior Scientist; Program Manager - Nitrogen Utilization Efficiency

- Manage a nitrogen utilization efficiency discovery and development project across crop species
- Responsible for development of project intellectual property
- Research project manager for licensing agreements in NUE

Renessen, LLC, Davis, CA **1999-2004**
(Cargill/Monsanto Joint Venture for Animal Feed and Grain Processing)

Technical Lead - Corn Oil (2001-2004)

- Managed a multi-million dollar research project on transgenic corn oil sponsored by the JV and performed at multiple research sites.
- Authored and monitored workplans and project budgets with investor R&D organizations.
- Coordinated discovery plans with the processing, animal nutrition and commercial development groups and communicated progress and long-term strategy to JV management and investors.
- Responsible for development of project intellectual property with attorneys and monitoring freedom to operate.
- Developed and managed third party technical relationships including consultants and contracts.

Crop Lead - Canola/Technical Lead - Oils (1999-2000)

- Initial employee of the joint venture at incorporation. Provided technical leadership for joint venture-sponsored canola projects.

- Managed crop discovery pipeline including targets on fiber, nutrition and oils.
- Assisted in prioritization of projects in tight financial climate and hiring of co-lead for oils.

Monsanto Co., Davis, CA **1997-1998**

Project Leader – Stearate Oils (1997-1998)

- Developed trans fatty acid-free oils for the Food and Nutrition Sector in both canola and soybean.
- Unified two stearate oils research projects with a common goal into one technical program across companies and sites.
- Developed comprehensive workplans for the project which integrated discovery phase technology with prototype development, plant breeding, food applications, preregulatory and business development.
- Initiated evaluation and prioritization of molecules for transgenic production of low calorie fats in plants.

Calgene, LLC, Davis, CA **1984-1996**

Research Project Leader – High Saturate Oils/Oils Division (1995-1996); Senior Scientist (1992-1994); Principal Scientist (1984-1991)

- Managed discovery phase high saturates oil composition research in canola.
- Coordinated research with breeding, food applications, product development and business development areas.
- Responsible for cloning and expressing plant genes to increase saturated fatty acid content of canola oil including desaturases, synthases and thioesterases.
- Discovered a unique thioesterase that increased stearate levels in transgenic plants.
- Member of the Nutrition and Consumer Products R&D Integration Team in the Monsanto acquisition of Calgene.
- Scientific management responsibility for research contract relationship with a multinational corporate client.
- Using an antisense strategy, demonstrated the first alteration of vegetable oil composition by genetic engineering.
- Discovered, patented and developed proprietary seed-specific promoters for canola gene expression.
- Other research programs included molecular plant virology, viral disease resistance, stress tolerance and general plant gene expression.
- Supervisory responsibility for BS and MS level research associates and training of student interns.
- Member of Calgene's research associate career development board.

University of Minnesota, St. Paul, MN **1981-1983**

Post-doctoral Research Associate, Laboratory of Dr. Joachim Messing

- Research on zein storage protein gene structure and plant tubulin genes.

EDUCATION

Ph.D. 1982 Biochemistry; University of California, Davis
Cowpea Mosaic Virus Coat Protein Structure Laboratory of Dr. George Bruening
B.A. 1975 Biochemistry and Molecular Biology; University of California, Santa Barbara

AWARDS AND HONORS

Phi Beta Kappa,1975, University of California Santa Barbara
American Cancer Society Institutional Research Grant, 1983

PATENTS, PUBLICATIONS and REVIEWS

PATENTS:

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Methods and Compositions for Regulated Transcription and Expression of Heterologous Genes. Vic. C. Knauf and **Jean C. Kridl** Issued: 8/28/01**US 6281410**; Issued: 11/9/99 **US 5981839**

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PUBLICATIONS:

Hawkins, D.J. and **J.C. Kridl** (1998) Characterization of acyl-ACP thioesterases of mangosteen (*Garcinia mangostana*) seed and production of high levels of stearate in transgenic canola seed oil. The Plant Journal 13:743-752.

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Kridl, J.C., D.W. McCarter, R.E. Rose, D.E. Scherer, D.S. Knutzon, S.E. Radke and V.C. Knauf. (1991) Isolation and characterization of an expressed napin gene from *Brassica rapa*. Seed Science Research 1:209-219.

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of a zein genomic clone with a short open reading frame. Gene 28:113-118.

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Exhibit 2

The Arabidopsis Book

First published on September 30, 2003; doi: 10.1199/tab.0010

Primary N-assimilation into Amino Acids in *Arabidopsis*

Gloria M. Coruzzi

Department of Biology, New York University, 100 Washington Square East, Room 1009 Main Building, New York, NY 10003

INTRODUCTION

The assimilation of inorganic nitrogen into organic form has marked effects on plant productivity, biomass, and crop yield (Hageman and Lambert, 1988; Lawlor et al., 1989). As such, a tremendous amount of biochemical and physiological studies have been performed on nitrogen assimilatory enzymes from a variety of plant species, especially crops and legumes. Summaries of such biochemical studies can be found in several comprehensive reviews (Miflin and Lea, 1976, 1977; Miflin, 1980; Miflin and Lea, 1980; Miflin and Lea, 1980, 1982; Miflin and Cullimore, 1984; Poulton et al., 1989). Although these biochemical studies have provided a solid groundwork, a complete picture of the N-assimilation process and its regulation in a single plant is still lacking for a number of reasons. The existence of multiple isoenzymes for each step in nitrogen assimilation has confounded biochemical purification as have the technical difficulties of isoenzyme purification and organelle isolation. As the mechanisms controlling intra- and intercellular transport of inorganic and organic nitrogen in plants are still under investigation, it is impossible to predict the *in vivo* function of nitrogen assimilatory enzymes localized in distinct cells or subcellular compartments based on *in vitro* biochemistry. Plant mutants have provided a mechanism to dissect the process of N-assimilation *in vivo* (Lea and Forde, 1994) (Lam et al., 1996). The aim of this chapter is to specifically highlight and update examples where molecular, genetic, and biochemical analyses of N-assimilation genes and mutants in *Arabidopsis* have begun to define the *in vivo* roles of individual isoenzymes in plant nitrogen assimilation and to uncover the mechanisms regulating this process.

In all higher plants, inorganic nitrogen is first reduced to ammonia prior to its incorporation into organic form (Lea, 1993). For a review of the regulation of Nitrate assimilation and reduction in *Arabidopsis* see (Crawford and Forde, 2002). Ammonia is assimilated into organic form as glutamine and glutamate, which serve as the nitrogen donors in the biosynthesis of essentially all amino acids, nucleic acids, and other nitrogen-containing compounds such as chlorophyll (Lea, 1993). The individual isoenzymes of GS, GOGAT or GDH have been proposed to play roles in three major ammonium assimilation processes: (i) primary nitrogen assimilation; (ii) reassimilation of photorespiratory ammonia; and (iii) reassimilation of "recycled" nitrogen. For a review of these processes see (Stewart et al., 1980; Lea, 1993). Glutamine and glutamate can then be used to form aspartate and asparagine, and these four amino acids are used to translocate organic nitrogen from sources to sinks (Lea and Miflin, 1980; Peoples and Gifford, 1993). The enzymes involved in the primary assimilation of ammonium into these four N-transport amino acids Glu/Gln and Asp/Asn are shown in Fig. 1 and include; glutamine synthetase (GS), glutamate synthase (GOGAT), aspartate aminotransferase (AAT) and asparagine synthetase (AS). While most studies of nitrogen metabolism have previously been performed in legumes and crop species, HPLC analyses of *Arabidopsis* has demonstrated that these same four amino acids can account for 60-64% of the total free amino acids present in *Arabidopsis* leaves and are also transported in the vascular tissues (Fig. 2) (Schultz, 1994; Lam et al., 1995). Thus, *Arabidopsis* appears to be a suitable model plant for the study of nitrogen assimilation into primary amino acids and the results should have impact on less genetically tractable crop plants. It is noteworthy that each enzyme for GS, GOGAT, AspAT and AS exists as multiple isoenzymes, encoded by multiple genes, even in *Arabidopsis* (Fig. 1). Table 1 lists the enzymes, the genes, and available mutants for these N-assimilatory enzymes in *Arabidopsis*. Below, we review how studies of the genes and mutants for these isoenzymes in *Arabidopsis* have helped to illuminate the role of specific genes in this N-assimilation pathway.

Glutamine Synthetase (Gs, E.C.6.3.1.2)

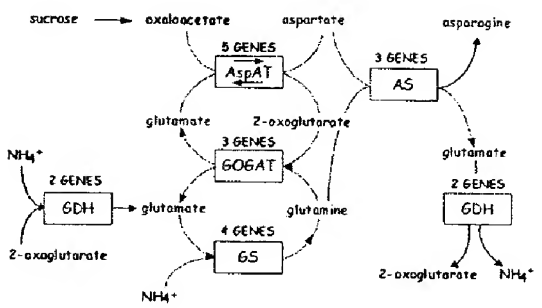


Figure 1. Nitrogen Assimilatory Pathway

GS, which has a very high affinity for ammonia (Km 3-5 μM), catalyzes the ATP-dependent conversion of glutamate into glutamine by incorporating a molecule of ammonia (Lea et al., 1990). GS is an octomeric holoenzyme, and two classes of GS isoenzymes were originally identified by ion-exchange chromatography: cytosolic GS1 and chloroplastic GS2 (Lea et al., 1990). The distinct physiological roles of chloroplast GS2 and cytosolic GS1 has been suggested primarily based on their organ-specific distribution. For example, as chloroplastic GS2 is the predominant isoenzyme in leaves, it has been proposed to function in primary assimilation of ammonia reduced from nitrate in chloroplasts and/or in the reassimilation of photorespiratory ammonia (Miflin and Lea, 1980). As cytosolic GS1 is the predominant isoenzyme in roots, it has been proposed to function in root nitrogen assimilation, although root plastid GS2 has also been implicated in this process (Miflin, 1974). The expression and localization of GS1 in vascular bundles of number of species further supports the notion that cytosolic GS1 functions to generate glutamine for intercellular nitrogen transport (Edwards and Coruzzi, 1990; Carvalho et al., 1992; Kamachi et al., 1992). This data, along with molecular-genetic studies described below in *Arabidopsis* have helped to assign *in vivo* functions to each GS isoenzyme.

Arabidopsis like all other higher plants contains a single nuclear gene for chloroplast GS2 (GLN2) and multiple genes for cytosolic GS1 (GLN1) (Peterman and Goodman, 1991; Oliveira and Coruzzi, 1999). Gene expression studies in *Arabidopsis* have shown that GLN2 is expressed predominantly in leaves, and its expression is regulated by light via phytochrome (Peterman and Goodman, 1991; Oliveira and Coruzzi, 1999). By contrast, the GLN1 genes (GLN1.1-1.3) encoding cytosolic GS1 isoenzymes are expressed at higher levels in roots (Oliveira and Coruzzi, 1999) (Peterman and Goodman, 1991). These organ-specific expression patterns suggest that GLN2 functions in leaf specific processes such as primary N-assimilation, and photorespiration. By contrast, the cytosolic GS1 isoenzymes are likely to function in primary N-assimilation in roots, as N is exported from

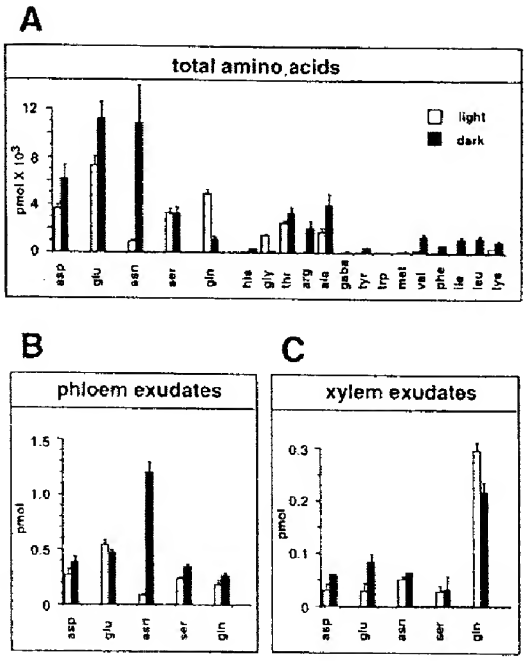


Figure 2. Amino Acid Levels in Light-Grown and Dark-Adapted Wild-Type *Arabidopsis* Plants. Amino acids levels in *Arabidopsis* plants grown in light (empty boxes) or subsequently dark adapted for 24 hr (filled boxes). The standard three letter code is used for all amino acids. **(A)** Average total free amino acid content. Each sample represents the average of three different plants (two leaves/plant). Gaba, γ-amino butyric acid. **(B)** Average free amino acid content in phloem exudates of three independent plants (one leaf/plant). **(C)** Average free amino acid content of xylem sap collected from cut hypocotyls of three independent plants. Data are from Schultz (1994). From: Hon-Ming Lam, Karen Coschigano, Carolyn Schultz, Rosana Melo-Oliveira, Gabrielle Tjaden, Igor Oliveira, Nora Ngai, Ming-Hsiun Hsieh, and Gloria Coruzzi (1995). Use of *Arabidopsis* Mutants and Genes to Study Amide Amino Acid Biosynthesis. Plant Cell 7, 887-898.

roots via xylem as Gln (Fig. 2C). Gene expression studies also showed regulation of GS mRNA levels by light, carbon and nitrogen metabolites (Oliveira and Coruzzi, 1999). In particular, GS expression is induced by light or sucrose and repressed by applications of Glu and Gln (Fig. 3A). These gene regulation patterns mimic changes in levels of GS enzyme activity (Oliveira and Coruzzi, 1999) (Fig. 3B). This analysis suggests that N-assimilation into Gln is active when levels of carbon backbones are high (in the light), and is repressed when levels of Gln are high (Oliveira and Coruzzi, 1999; Oliveira et al., 2001). This negative regulation of GS expression by levels of Glu/Gln is reminiscent of the Ntr system of GS in bacteria (Magasanik, 1994). In fact, a component of the Ntr system in bacteria (PII) has been identified in *Arabidopsis*, and there is some evidence that it may be involved in sensing C:N ratios, and in regulating N-assimilation in

Table 1. N-assimilation-Enzymes, Genes, Mutants.

Amino Acid	Enzyme	Gene Name	Gene ID#	Organelle	Mutant	References
Glutamine	GS glutamine synthetase	*GLN 2*	At5g35630	Chloroplastic		Peterman and Goodman (1991)
		GLN 1.1	At5g37600	Cytosolic		Oliveira *et al* (2002)
		GLN 1.2	At1g66200	Cytosolic		
		GLN 1.3	At3g17820	Cytosolic		
Glutamate	Fd-GOGAT glutamate synthase	*GLU 1*	At5g04140	Chloroplastic	gls 1	Somerville and Ogren (1980)
		GLU 2	At2g41220	Chloroplastic		Coschigano *et al* (1998)
	NADH-GOGAT glutamate synthase	*GLT 1*	At5g53460	Chloroplastic	glt 1T	Lancien *et al* (2002)
	GDH glutamate dehydrogenase	*GDH 1*	At5g18170	Mitochondrial	gdh 1-1	Melo-Oliveira *et al* (1996)
		GDH 2	At5g07440	Mitochondrial		Turano *et al* (1997)
Aspartate	AAT aspartate asminotransferase	*ASP 1*	At2g30970	Mitochondrial		Schultz *et al* (1995, 1998)
		ASP 2	At5g19550	Cytosolic	aat 2	Miesak and Coruzzi (2002)
		ASP 3	At5g11520	Peroxisomal		
		ASP 4	At1g62800	Cytosolic		Schultz *et al* (1995, 1998)
		ASP 5	At4g31990	Chloroplastic	aat 3	Wilkie *et al* (1995)
Asparagine	ASN asparagine synthetase	*ASN 1*	At3g47340	Cytosolic		Lam *et al* (1994, 1998, 2003)
		ASN 2	At5g65010	Cytosolic		Lam *et al* (1998)
		ASN 3	At5g10240	Cytosolic		

*All mutant strains are available through ABRC.

plants (Hsieh et al., 1998; Smith et al., 2002; Smith et al., 2003).

Regarding mutant screens in *Arabidopsis*, thus far no GS mutants have been reported. This is surprising, as GS mutants have been uncovered in photorespiratory mutants screens in other species (barley) (Wallsgrove et al., 1987). While identical screens for plant mutants unable to survive in photorespiratory conditions were conducted in *Arabidopsis* (Somerville and Ogren, 1980) mutants specifically defective in GS2 were identified only in the barley screen (Wallsgrove et al., 1987). A dramatic result of this mutant study in barley is the finding that a GS2 isoenzyme located in the chloroplast is essential for the reassimilation of photorespiratory ammonia that is released in mitochondria. Paradoxically, the barley GS2 mutants were unable to reassimilate photorespiratory ammonia released in the mitochondria even though they contained normal levels of cytosolic GS1 (Wallsgrove et al., 1987). This paradox has been resolved by studies on the cell-specific expression patterns of genes for chloroplastic GS2 and cytosolic GS1. Studies of GS-promoter-GUS fusions revealed that chloroplastic GS2 and cytosolic GS1 of pea are expressed in non-overlapping cell types in transgenic tobacco (Edwards et al., 1990). Chloroplastic GS2 is expressed predominantly in leaf mesophyll cells, where photorespiration occurs, while cytosolic GS1 is expressed exclusively in the phloem (Forde et al., 1989; Edwards et al., 1990). These promoter-GUS fusion results were later confirmed by studies of the native cytosolic GS1 proteins in rice and tobacco (Carvalho et al., 1992; Kamachi et al., 1992). In those studies, antibodies specific to cytosolic GS1 were used in *in situ* immunolocalization experiments to show that all cytosolic GS1 proteins are expressed solely in vascular tissues. Thus, this vascular-specific expression pattern may explain why cytosolic GS1 cannot compensate for the loss of chloroplastic GS2 in mesophyll cells of the barley GS2 mutants.

One piece of the GS isoenzyme puzzle that is outstanding is fact that the screens for photorespiratory mutants in *Arabidopsis* failed to uncover any mutants defective in GS, either chloroplastic GS2 or cytosolic GS1. There are several possible explanations for this finding: (i) The *Arabidopsis* photorespiratory screen was not saturating. This is unlikely as multiple alleles for many enzymes in the photorespiratory pathway were isolated in that screen, including 58 mutants affecting Fd-GOGAT (Somerville and Ogren, 1980; Artus, 1988). (ii) Both chloroplastic GS2 and cytosolic GS1 are expressed in mesophyll cells, so that a mutation in one gene is masked. (iii) There is more than one gene for chloroplastic GS2 in *Arabidopsis*. This is not possible as the complete sequence of the *Arabidopsis* genome (Bevan et al., 2001) has revealed a single gene for chloroplastic GS2. (iv) A mutation in chloroplastic or cytosolic GS is lethal in *Arabidopsis*, preventing the isolation of mutants.

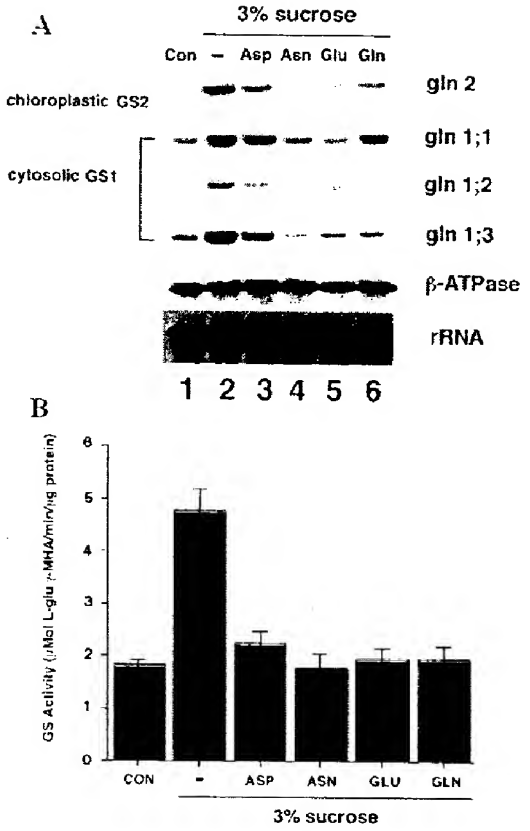


Figure 3. C:N Regulation of GS mRNA levels and GS Activity. **(A)** Amino acids and carbon reciprocally regulate GS mRNA accumulation and enzyme levels. *Arabidopsis* plants were grown semihydroponically and dark-adapted. The dark-adapted *Arabidopsis* plants were transferred in the dark to fresh low-nitrogen MS medium with no carbon supplementation (Con, lane 1), to fresh low-nitrogen MS medium with 3% Suc (lane 2), or to fresh low-nitrogen MS medium with 3% Suc in addition of either 10 mM Asp (lane 3), 10 mM Asn (lane 4), 10 mM Glu (lane 5), or 10 mM Gln (lane 6). After transfer, the plants were further incubated for 12 h in the dark. **(B)** Same as A except that an aliquot of each sample was collected for determination of total GS activity. A representative experiment of two repetitions is shown (results are GS activity per milligram of total protein ± SE of three independent determinations). From: Igor C. Oliveira and Gloria M. Coruzzi (1999). Carbon and Amino Acid Reciprocally Modulate the Expression of Glutamine Synthetase in *Arabidopsis*. Plant Physiology 121, 301-309.

Glutamate Synthase/ (Nadh-Gogat: E.C.1.4.1.14; FD-Gogat: E.C.1.4.7.1)

Glutamate synthase (GOGAT) functions in a cycle with glutamine synthetase (GS), catalyzing the transfer of the amide group from glutamine (generated by GS) to 2-oxoglutarate to form two molecules of glutamate (Fig. 1).

Table 2. Relative GOGAT activity levels in wild-type and Fd-GOGAT mutants (gls1).

	Fd-GOGAT	NADH-GOGAT	Total GOGAT
WT	95%	5%	100%
gls1	5%	5%	10%

In higher plants, there are two forms of GOGAT, a Fd- and an NADH-dependent GOGAT, each of which is chloroplast localized (Lea, 1993). Fd-GOGAT is uniquely found in photosynthetic organisms and biochemical studies in *Arabidopsis* have shown that Fd-GOGAT is the major isoenzyme in leaves accounting for 95% of the GOGAT activity, while NADH-GOGAT activity is a minor component in leaves accounting for 5% (Somerville and Ogren, 1980) (Table 2). These organ-specific distribution patterns were used to infer *in vivo* function. For Fd-GOGAT, the high levels in leaves suggested that it plays a role in N-assimilation processes in photosynthetic tissues such as primary N-assimilation and/or photorespiration. By contrast, biochemical studies on other species showed that NADH-GOGAT is located primarily in plastids of non-photosynthetic tissues such as roots (Matoh and Takahashi, 1982; Suzuki and Gadal, 1984) where it is proposed to function in primary assimilation or reassimilation of ammonia released during amino acid catabolism (Miflin and Lea, 1980). The molecular and genetic studies of GOGAT genes and mutants in *Arabidopsis* outlined below have helped to clarify the relative *in vivo* roles of Fd- vs. NADH-GOGAT in primary N-assimilation vs. the reassimilation of photorespiratory ammonium.

Arabidopsis mutants in Fd-GOGAT were uncovered in 1980 during a screen for photorespiratory mutants (Somerville and Ogren, 1980). In the three *Arabidopsis* gluS (aka gls) mutants initially characterized, leaf Fd-GOGAT activity was reduced to less than 5% of wild type levels, whereas NADH-GOGAT - which contributes about 5% of the total GOGAT activities in normal conditions- remained unchanged (Somerville and Ogren, 1980) (Table 2). The gluS mutants had a conditional phenotype: they were chlorotic when grown in air (photorespiratory conditions), but could be rescued when grown in conditions under which photorespiration was suppressed (1% CO2) (Fig. 4). This finding suggested that Fd-GOGAT plays a major role in the reassimilation of photorespiratory ammonium released in mitochondria. This gluS mutant revealed a number of surprises that could not have been determined based on traditional biochemical studies. 1. It showed that photorespiratory ammonium released in the mitochondria is reassimilated primarily in the chloroplast by Fd-GOGAT. Thus, despite the fact that glutamate dehydrogense (GDH) is located in the mitochondria, these studies on the gluS mutants suggested GDH plays a minor role, if any, in the reassimi-

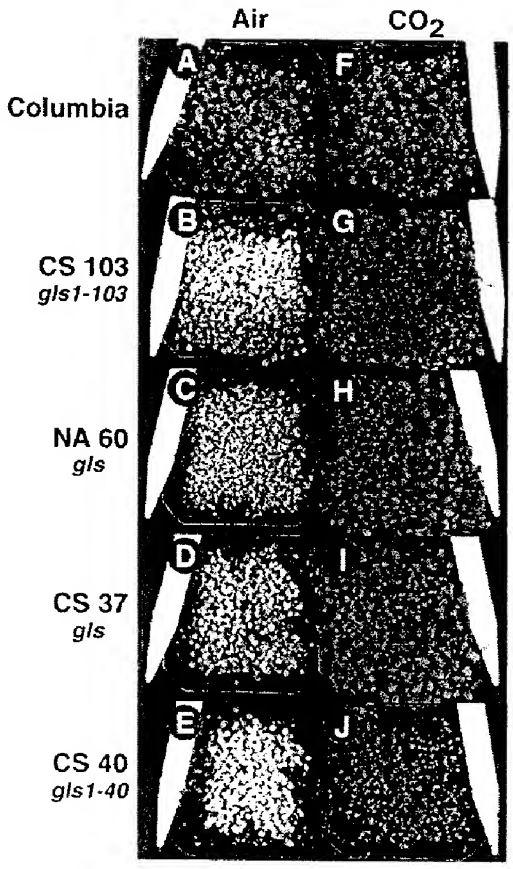


Figure 4. Photorespiratory Phenotype of *gls* Mutants Defective in Fd-GOGAT Activity. In **(A)** - **(E)**, *Arabidopsis* plants were grown in air; in **(F)** - **(J)**, plants were grown in 2% CO_2. Genotypes: **(A)** and **(F)** *Arabidopsis* wild-type Columbia. **(B)** and **(G)** Photorespiratory *gls* mutant CS103. **(C)** and **(H)** NA60. **(D)** and **(I)** CS37. **(E)** and **(J)** CS340. From: Karen T. Coschigano, Rosana Melo-Oliveira, Jackie Lam, and Gloria Coruzzi (1998). *Arabidopsis gls* Mutants and Distinct Fd-GOGAT Genes: Implications for Photorespiration and Primary Nitrogen Assimilation. Plant Cell 10, 741-752.

lation of photorespiratory ammonium. 2. The gluS mutant studies showed that Fd-GOGAT plays a major role in photorespiration, and that the normal low levels of NADH-GOGAT in leaves could not compensate for the loss of Fd-GOGAT. 3. The finding that the gluS mutants grew normally in high CO_2 (non-photorespiratory conditions) was paradoxical, and suggested that Fd-GOGAT, the major GOGAT enzyme in leaves, was dispensable in primary N assimilation. Molecular analysis of the genes for Fd-GOGAT in *Arabidopsis*, and further studies of the gluS mutants helped to clarify this latter point. Coschigano et al identified two expressed genes for Fd-GOGAT from *Arabidopsis*, GLU1 and GLU2 (Coschigano et al., 1998). This was paradoxical, as the gluS mutants suggested the existence of a single genetic locus for Fd-GOGAT. Further studies resolved this issue. Gene expression studies showed that GLU1 was the major expressed gene in leaves, and that its mRNA was light regulated. By contrast, GLU2 was expressed primarily in roots (Fig. 5). Studies further showed that GLU1 is genetically linked to the gluS mutation, while GLU2 mapped to a separate chromosome (Coschigano et al., 1998). Thus, it appears that mutations in the highly expressed GLU1 gene are able to cause a mutant photorespiratory phenotype, and that the low levels of Fd-GOGAT from GLU2, and the normally low levels of NADH-GOGAT in leaves are unable to compensate for the loss of GLU1, encoding the major Fd-GOGAT isoenzyme in *Arabidopsis*. A similar situation has been seen in the *Arabidopsis* pathway of tryptophan synthesis, where a mutation in a highly expressed gene for tryptophan synthase leads to auxotrophy, even though a second less highly expressed tryptophan synthase gene was unaffected (Last et al., 1991).

The fact that all of the Fd-GOGAT deficient mutants isolated were chlorotic and eventually died when grown in atmospheric conditions promoting photorespiration (air), established an essential role for Fd-GOGAT in photorespiration. However, since the Fd-GOGAT deficient mutants recovered and were viable when grown in conditions where photorespiration was suppressed (high carbon dioxide or low oxygen), Fd-GOGAT appeared

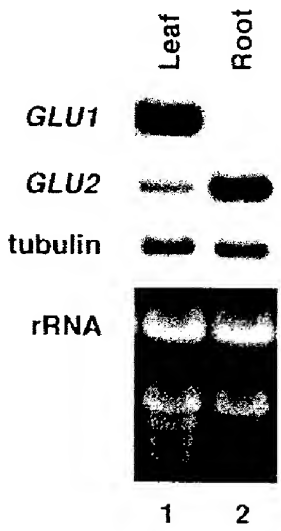
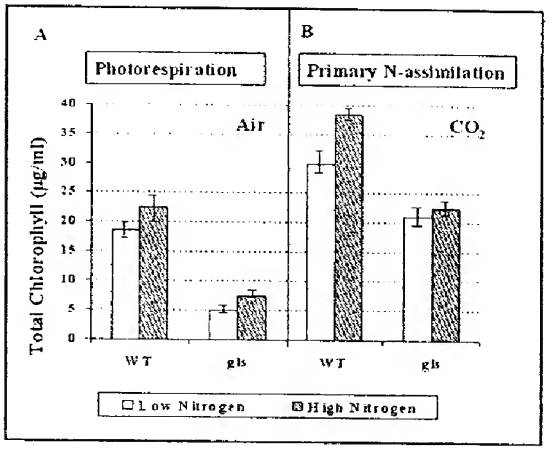


Figure 5. Levels of mRNA for Fd-GOGAT genes *GLU1* & *GLU2* in leaves vs. roots. GLU1 is the major mRNA in leaves, while GLU2 predominates in roots. Control RNA is tubulin. From: Karen T. Coschigano, Rosana Melo-Oliveira, Jackie Lam, and Gloria Coruzzi (1998). *Arabidopsis gls* Mutants and Distinct Fd-GOGAT Genes: Implications for Photorespiration and Primary Nitrogen Assimilation. Plant Cell 10, 741-752.



C

Effect	Air P	CO_2 P
1-WT vs. gls (NA60)	<0.0001 *	<0.0001 *
2-low vs. high nitrogen	0.0145 *	0.0005 *
3-1 x 2 interaction	0.7029	0.0191 *

Figure 6. A gls Mutant Displays a Defect in the Assimilation of Inorganic Nitrogen under Nonphotorespiratory Conditions. (A) and **(B)** Plants grown in air and plants in 1% CO_2 respectively. Wild-type Columbia (WT) and a *gls* mutant (NA60) were grown for 12 days on nitrogen-free MS media supplemented with either low levels of inorganic nitrogen (2 mM ammonium and 4 mM nitrate; open bars) or high levels of inorganic nitrogen (20 mM ammonium and 40 mM nitrate; hatched bars). Mean values of total chlorophyll measurements ± SE are shown. **(C)** Two-way ANOVA of (1) the wild type (WT) versus a *gls* mutant in air and CO_2 (2) low versus high nitrogen conditions in air and CO_2, and (3) interactions of 1 and 2 as given above. Statistically significant results are indicated by an asterisk. From: Karen T. Coschigano, Rosana Melo-Oliveira, Jackie Lam, and Gloria Coruzzi (1998). *Arabidopsis gls* Mutants and Distinct Fd-GOGAT Genes: Implications for Photorespiration and Primary Nitrogen Assimilation. Plant Cell 10, 741-752.

at first glance to be dispensable for non-photorespiratory roles, such as in primary nitrogen assimilation. This conclusion was paradoxical as most primary assimilation occurs in leaves, where Fd-GOGAT activity predominates (95% of total GOGAT activity) and NADH-GOGAT is a minor component (5% of total GOGAT activity) (Somerville and Ogren, 1980). How could Fd-GOGAT, the major GOGAT activity in leaves be dispensable for primary nitrogen assimilation? To address this question, Coschigano et al measured levels of chlorophyll as an indicator of N-status (Delgado et al., 1994) in wild-type and gluS mutant plants grown under non-photorespiratory conditions (Fig. 6). These studies suggest that the primary gluS mutants in fact have defects in N-assimilation (Coschigano et al., 1998). However, the gluS mutants are viable, suggesting that the normal low level

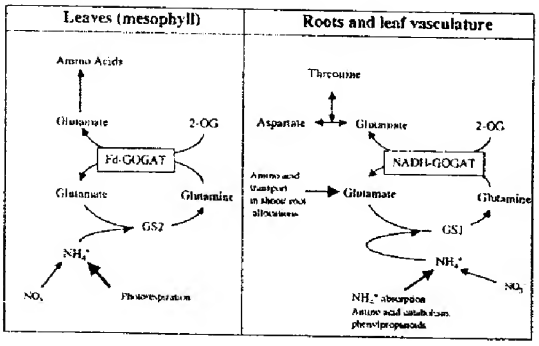


Figure 7. A Model for the Non-Redundant Roles of GOGAT Isoenzymes in Leaves and Roots. Model based on analysis of *Arabidopsis* GOGAT mutants in Fd-GOGAT (gls) or NADH-GOGAT (glt1-T) (Somerville and Ogren, 1980), (Coschigano et al., 1998), (Lancien et al., 2002). Fd-GOGAT in leaves encoded by Glu1 is primarily responsible for assimilation of photorespiratory ammonia while NADH-GOGAT is responsible for glutamate synthesis in roots. From: Muriel Lancien, Melinda Martin, Ming-Hsiun Hsieh, Tom Leustek, Howard Goodman and Gloria M. Coruzzi (2002). *Arabidopsis glt1*-T mutant defines a role for NADH-GOGAT in the non-photorespiratory ammonium assimilatory pathway. Plant Journal 29(3), 347-358.

expression of GLU2 in the mutants (and potentially NADH-GOGAT) is sufficient to support primary N-assimilation.

In contrast to Fd-GOGAT, NADH-GOGAT appears to be present primarily in non-green tissues such as roots. *Arabidopsis* has a single gene for NADH-GOGAT, GLT1, and recently, a T-DNA knock out mutant in this gene has been identified (Lancien et al., 2002). The GLT1 gene is expressed at low constitutive levels in leaves, and at higher levels in roots. The T-DNA mutant in the GLT1 gene (glt1-T) was null, and had no detectable levels of NADH GOGAT (Lancien et al., 2002). The glt1-T mutant was thus used to study the in vivo role of NADH GOGAT by scoring the mutants for defects related to N-assimilation, compared to wild-type and to a mutant in Fd-GOGAT (gls113) (Lancien et al., 2002). This analysis showed that the glt1-T mutant shows defects in fresh weight and chlorophyll accumulation, and glutamate production specifically when the plants are grown under non-photorespiratory conditions. These findings suggest that NADH-GOGAT plays a significant role in primary nitrogen assimilation in plants grown under non-photorespiratory conditions. The models for the respective roles of NADH vs. Fd-GOGAT are shown in Fig. 7.

Glutamate Dehydrogenase (Nadh-Gdh: E.C.1.4.1.2; Nadph-Gdh: E.C.1.4.1.4)

Glutamate dehydrogenase (GDH) is an enzyme that can catalyze forward and reverse biochemical reactions: the

amination of 2-oxoglutarate into glutamate (biosynthetic) or the deamination of glutamate into ammonia and 2-oxoglutarate (catabolic) (Fig. 1) (Lea et al., 1990). Two major forms of GDH have been reported, an NADH-dependent form (NADH-GDH) that is found in the mitochondria (Day et al., 1988; Loulakakis and Roubelakis-Angelakis, 1990), and an NADPH-dependent form (NADPH-GDH) localized to the chloroplast (Lea and Thurman, 1972). GDH is a hexameric enzyme (Srivastava and Singh, 1987). In *Arabidopsis*, the GDH enzymes can be resolved into seven isoenzymes by enzyme activity staining of non-denaturing gel electrophoresis (Cammaerts and Jacobs, 1983; Melo-Oliveira et al., 1996) (Fig. 8). These seven GDH activity bands are the result of the random association of two types of subunits into a hexameric complex. The most anodal homohexamer (GDH1) is thought to catalyze the anabolic reaction, while the cathodal most homohexamer (GDH2) is thought to be primarily catabolic (Cammaerts and Jacobs, 1985). Under this model, the heterohexamers of GDH1 and GDH2 would have intermediate catabolic and anabolic activities depending on the proportion of GDH1 versus GDH2 subunits. It has been proposed that two non-allelic genes are responsible for the synthesis of the GDH1 and GDH2 subunits (Cammaerts and Jacobs, 1985), and the discovery of two GDH genes: GDH1 and GDH2, and gdh1-1 mutant of *Arabidopsis* supports this conclusion, as discussed below (Melo-Oliveira et al., 1996; Turano et al., 1997). GDH isoenzymes have also been used to study the effect of nitrogen on GDH in *Arabidopsis* (Jacobs and Joukas, 1978)

Although GDH exists in plant tissues at high levels, there is an ongoing debate as to its physiological role in higher plants. Originally, GDH was proposed to be the primary route for the assimilation of ammonia in plants. However,

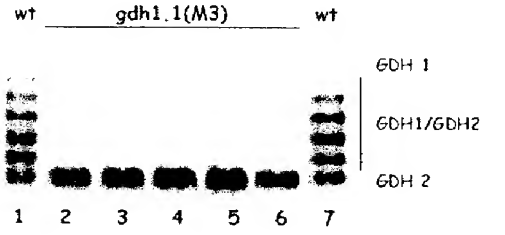


Figure 8. GDH Activity in Wild-Type *Arabidopsis* and *gdh* 1-1 Mutant. Crude leaf protein extracts were made from rosette leaves of 21-day-old *Arabidopsis* plants, separated by electrophoresis on a nondenaturing polyacrylamide gel, and stained for GDH activity. Lanes 1 and 7, extract of wild-type *Arabidopsis* (Columbia). The seven holoenzymes result from the formation of two homohexamers (GDH1 and GDH2), and five heterohexamers of GDH are indicated on the right (GDH1/GDH2). M_3 individuals from a selfed *gdh 1-1* mutant display only the GDH2 homohexamer (lanes 2-6). From: Rosana Melo-Oliveira, Igor Cunha Oliveira, and Gloria M. Coruzzi (1996). Arabidopsis mutant analysis and gene regulation define a nonredundant role for glutamate dehydrogenase in nitrogen assimilation. Proc. Natl. Acad. Sci. 93, 4718-4723.

this biosynthetic role of GDH has been challenged by the discovery of an alternative pathway for ammonia assimilation via the GS/GOGAT cycle (Miflin and Lea, 1976). Moreover, the fact that the GDH enzyme has a high Km for ammonia argues against a role in primary nitrogen assimilation (Stewart et al., 1980). Studies have shown that GDH enzyme activity can be induced in plants exposed to high levels of ammonia, and as such GDH has been proposed to be important specifically for ammonia-detoxification purposes. Mitochondrial GDH has also been proposed to be involved in the assimilation of high levels of photorespiratory ammonia released in mitochondria (Yamaya and Oaks, 1987). However, the isolation of photorespiratory mutants defective in chloroplastic GS2 (in barley) (Wallsgrove et al., 1987) or Fd-GOGAT (in barley and in *Arabidopsis*) (Somerville and Ogren, 1980; Kendall et al., 1986; Blackwell et al., 1987) have argued against the importance of GDH in photorespiration (Wallsgrove et al., 1987). Furthermore, treatment of plants with the GS inhibitor MSO prevents the incorporation of ammonia into glutamate and glutamine, even though both GDH activity and ammonia levels remain high (Lea et al., 1990). Altogether these results argue against a biosynthetic role for GDH. Instead a catabolic role for GDH has been invoked. Biochemical evidence for such a role is the fact that GDH activity is induced during germination and senescence, two periods where amino acid catabolism occurs (Stewart et al., 1980; Lea et al., 1990). To date, however, biochemical studies have failed to uncover the true *in vivo* role for GDH. The *Arabidopsis* gdh mutants described below have helped to define an *in vivo* role for GDH in plants.

Arabidopsis contains two genes for GDH: GDH1 and GDH2 (Melo-Oliveira et al., 1996; Turano et al., 1997). The predicted protein sequences suggest that they each encode NADH-dependent enzymes that are likely to be associated with the mitochondria. Gene expression studies have shown that each gene is subject to regulation by light and metabolites (Turano et al., 1997). *Arabidopsis* mutants deficient in GDH were identified in the M2 generation of EMS- and NMU-mutagenized *Arabidopsis* in a brute force screen using a GDH activity stain on crude leaf protein extracts following electrophoresis on native gels (Melo-Oliveira et al., 1996). A single *Arabidopsis* GDH mutant, gdh1-1, has been identified which has an altered pattern of GDH activity: it possesses a single GDH2 holoenzyme and is missing the GDH1 holoenzyme as well as the GDH1:GDH2 heterohexamers (Fig. 8) (Melo-Oliveira et al., 1996). The *Arabidopsis* gdh1-1 mutant displays an impaired growth phenotype compared to wild-type, specifically when plants are grown under conditions of high inorganic nitrogen (Fig. 9). This conditional phenotype suggests a non-redundant role for GDH in the assimilation of ammonia under conditions of inorganic nitrogen excess. A similar GDH-deficient mutant has been previously described in *Zea mays*, a C4 plant, which also appears to

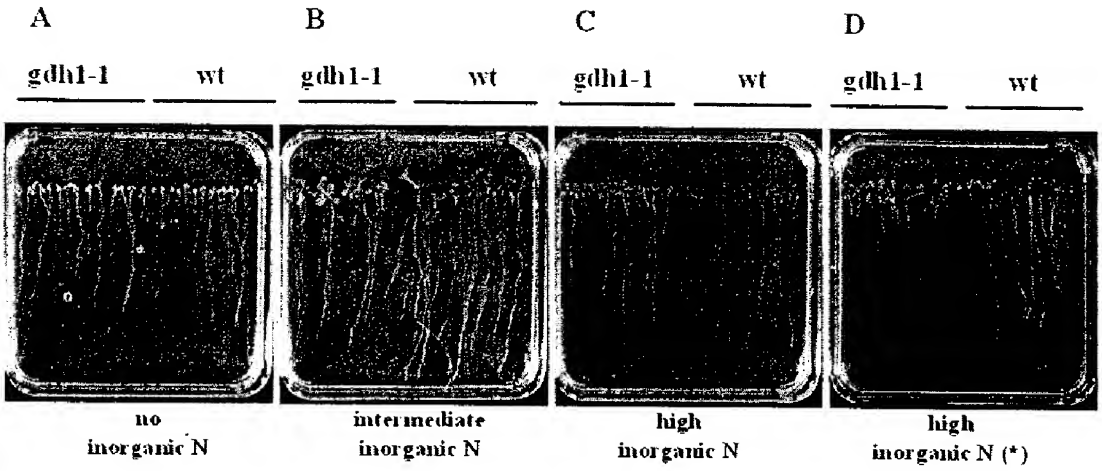


Figure 9. Growth Phenotype of the *gdh 1-1* Mutant. Growth of wild-type *Arabidopsis* versus the *gdh 1-1* mutant seedlings was measured in a vertical root length assay. Wild-type (wt) and M_3 seeds of the *gdh 1-1* mutant were sown side-by-side on ammonia-free/nitrate-free MS media containing vitamins and 3% sucrose supplemented with either: **(A)** no organic nitrogen (0 mM ammonia, 0 mM nitrate); **(B)** intermediate levels of inorganic nitrogen (2 mM ammonia, 4 mM nitrate); or **(C)** high levels of inorganic nitrogen (20 mM ammonia, 40 mM nitrate). **(D)** Plants grown on MS media supplemented with 3% sucrose containing high levels of inorganic nitrogen (20 mM ammonia, 40 mM nitrate) without the vitamin supplement (*). Plates were incubated vertically for 12 days and grown under a normal day/night cycle. From: Rosana Melo-Oliveira, Igor Cunha Oliveira, and Gloria M. Coruzzi (1996). *Arabidopsis* mutant analysis and gene regulation define a nonredundant role for glutamate dehydrogenase in nitrogen assimilation. Proc. Natl. Acad. Sci. 93, 4718-4723.

be affected in the GDH1 gene product (Pryor, 1974, 1979). Preliminary studies showed that the maize GDH mutant displays a growth phenotype only under low night temperatures (Pryor, 1990). Moreover, it has been reported that the maize GDH1 mutant shows a 10 to 15-fold lower total GDH activity when compared to wild type maize (Magalhaes et al., 1990). As the photorespiratory rate is very low or non-existent in a C4 plant, the maize GDH1 mutant cannot be used to assess the role of GDH in photorespiration. Therefore, the *Arabidopsis* gdh1-1 mutant will be valuable to assess the function of this enzyme in photorespiration in a C3 plant. It should be noted that neither the maize nor the *Arabidopsis* gdh mutants are null for GDH, as they each possess a second GDH2 gene.

Aspartate Aminotransferase (Aspat: E.C. 2.6.1.1)

Aspartate aminotransferase (AspAT) is a pyridoxal phosphate-dependent enzyme that catalyzes the reversible transfer of an amino group from glutamate to oxaloacetate to generate aspartate and 2-oxoglutarate (Fig. 1). The AspAT holoenzyme is a homodimer consisting of two subunits (Lea, 1993). In addition to its role in aspartate synthesis and catabolism, AspAT plays an important role in C3 plants, as part of the malate-aspartate shuttle which allows the transfer of reducing equivalents from mitochondria and chloroplasts into the cytoplasm (Ireland and Joy, 1985). Accordingly, biochemical studies show that AspAT exists as distinct isoenzymes found in different subcellular locations such as the cytosol, mitochondria, chloroplasts, glyoxysomes or peroxisomes. For examples see: (Weeden and Gottlieb, 1980; Wadsworth et al., 1993; Schultz and Coruzzi, 1995; Taniguchi et al., 1995). The subcellular compartmentation of AspAT isoenzymes suggests that the different forms of AspAT might serve distinct roles in plant metabolism. The isolation of *Arabidopsis* mutants in genes for distinct AspAT isoenzymes has helped to elucidate the role of these isoenzymes in plant N-assimilation.

Arabidopsis contains a family of five genes encoding distinct AspAT isoenzymes, ASP1-5 (Schultz and Coruzzi, 1995; Wilkie et al., 1995). ASP1 encodes mitochondrial AAT1, ASP2 and ASP4 encode cytosolic isoenzymes, ASP5 encodes chloroplastic AAT3, and ASP3 encodes a putative peroxisomal isoenzyme (Fig. 10) (Schultz and Coruzzi, 1995; Wilkie et al., 1995; Schultz et al., 1998). AAT isoenzymes of *Arabidopsis* were identified by native gel analysis. Three major isoenzymes were detected in crude leaf extracts, and identified by subcellular fractionation (Schultz and Coruzzi, 1995; Schultz et al., 1998). The two major isoenzymes are AAT2 (cytosolic) and AAT3 (chloroplastic) (Fig.10). Mitochondrial AAT1 was also detected when the gels were overloaded. This native gel assay was used in a brute force screen to identify plant mutants deficient in one of the two major AAT isoenzymes; cytosolic AAT2 and chloroplastic AAT3

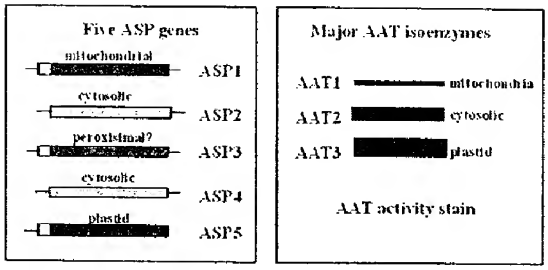


Figure 10. ASP Genes and Major AAT Isoenzymes in *Arabidopsis*. Five ASP genes encoding AAT isoenzymes: ASP1 encodes mitochondrial AAT1, ASP2 encodes cytosolic AAT2, ASP5 encodes chloroplastic AAT3, ASP3 encodes a putative peroxisomal AAT, and ASP4 encodes a minor cytosolic AAT.

(Fig. 11) (Schultz et al., 1998; Miesak and Coruzzi, 2002), as described below.

To help determine the *in vivo* function(s) of each AspAT isoenzyme, a screen for *Arabidopsis* mutants defective in either cytosolic AAT2 or chloroplastic AAT3 was performed. M2 seedlings were screened for alterations in AAT profiles using the native gel screen on crude leaf extracts (Schultz et al., 1998; Miesak and Coruzzi, 2002). Screens of EMS-mutagenized *Arabidopsis* seeds and T-DNA lines uncovered two genetic loci: aat2 mutants defective in cytosolic AAT2, and aat3 mutants defective in chloroplastic AAT3 (Fig. 11). The ASP genes and aat mutants were mapped and the defective ASP genes sequenced in the aat mutants (Schultz et al., 1998; Miesak and Coruzzi, 2002). By analyzing the effects of these mutations on the growth phenotypes and the free amino acid pools in the aat mutant plants, the *in vivo* importance of each AAT isoenzyme was determined. Initial analyses show that a mutation in the cytosolic ASP2 gene results in a retarded growth phenotype and a decrease in the pools of free aspartate (Schultz et al., 1998). Moreover, the aat2 mutants also showed dramatic decreases in asparagine in dark-adapted plants (Fig. 12). These studies indicate that cytosolic AAT2 is responsible for the synthesis of pools of aspartate in the light that are used for asparagine synthesis in the dark. Further analysis of additional aat2 alleles, including a T-DNA mutant in ASP2 revealed additional information on the cytosolic AAT2 isoenzyme (Miesak and Coruzzi, 2002). For example, in an attempt to determine whether the growth deficiency of aat2 was due to an aspartate deficiency, aat2 mutants were supplied with exogenous aspartate. Surprisingly, the aat2 mutants exhibited an aspartate sensitivity (compared to wild-type) suggesting that the aat2 mutation led not only to an inability to synthesize aspartate, but also an inability to catabolize aspartate (Miesak and Coruzzi, 2002). These studies indicate that cytosolic AAT2 plays a non-redundant function compared to the other 4 ASP genes. Further HPLC studies on siliques of aat2 mutants revealed deficiencies in Asp and Asn in siliques, suggesting that

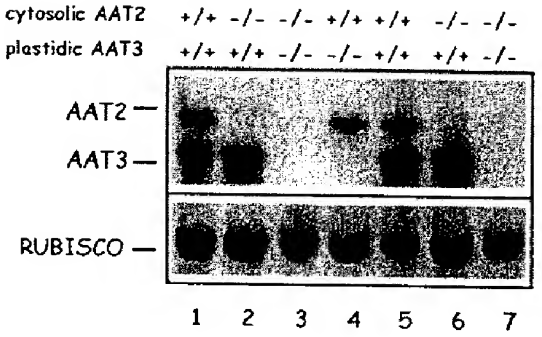


Figure 11. AspAT Activity Gels of Wild-Type, Single and Double *aat* Mutants. AspAT activity gel of seven different F_2 individuals from the cross *aat2-1/aat2-1* X *aat3-2/aat3-2* showing individuals which are homozygous wild-type for both cytosolic and chloroplastic activity (lanes 1 and 5); homozygous mutant for cytosolic AAT2 but wild-type for chloroplastic AAT3 (lanes 2 and 6); homozygous mutant for both cytosolic and chloroplastic AspAT (lanes 3 and 7); and wild-type for cytosolic AAT2 but homozygous mutants for chloroplastic AAT3 (lane 4). The genotypes of the F_2 individuals shown are as follows: *AAT2-1/AAT2-1, AAT3-2/AAT3-2* (lanes 1 and 5); *aat2-1/aat2-1, AAT3-2/AAT3-2* (lanes 2 and 6); *aat2-1/aat2-1, aat3-2/aat3-2* (lanes 3 and 7); *AAT2-1/AAT2-1, aat3-2/aat3-2* (lane 4). The amount of rubisco protein was determined by Coomassie staining of the gel after activity staining. From: Schultz CJ (1994) A molecular and genetic dissection of the aspartate aminotransferase isoenzymes of *Arabidopsis thaliana*. A Ph.D. thesis. New York University, New York, NY

cytosolic AAT2 plays an important role in N-transport to siliques (Miesak and Coruzzi, 2002).

Asparagine Synthetase (AS: E.C.6.3.5.4)

Asparagine was the first amino acid discovered, as it was crystallized from asparagus almost 200 years ago (Vauquelin and Robiquet, 1806). Despite this historical placement, the mechanism of asparagine biosynthesis in plants has been elucidated in plants only recently. While three possible routes for asparagine synthesis have been proposed (Sieciechowicz et al., 1988), the glutamine-dependent asparagine synthetase enzyme is now generally accepted as the major route for asparagine biosynthesis in plants (Lea et al., 1990). In an ATP-dependent reaction, AS catalyzes the transfer of an amino group of glutamine to a molecule of aspartate to generate a molecule of glutamate and asparagine (Fig. 1). Glutamine is the preferred substrate for nearly all of the AS enzymes studied in higher plants (Sieciechowicz et al., 1988). However, ammonia was also reported to be a possible AS substrate, particularly in the case of maize roots (Oaks and Ross, 1984). Traditional biochemical studies of AS in higher plants were hampered by the fact that AS is a very unstable enzyme *in vitro* and only partially purified AS enzymes have been isolated from

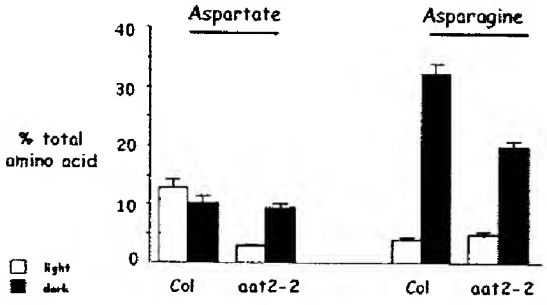


Figure 12. *aat2-2* mutants have specific reductions in levels of aspartate in light-grown plants and asparagine in dark-adapted plants. The relative proportions of aspartate and asparagine in the phloem exudates from wild-type Columbia (Col) and *aat2-2* mutant plants grown in light (unshaded boxes) or dark adapted (shaded boxes). Each sample is the average of a single leaf from three representative plants. Plants were grown in soil in a normal day/night cycle (16 hr light/8 hr dark) for 3 wk and either light adapted (unshaded box) or dark adapted (shaded box) for 24 hr. Error bars represent the standard error of the mean. From: Carolyn J. Schultz, Meier Hsu, Barbara Miesak and Gloria Coruzzi (1998). *Arabidopsis* Mutants Define an *in Vivo* Role for Isoenzymes of Aspartate Aminotransferase in Plant Nitrogen Assimilation. Genetics 149; 491-499.

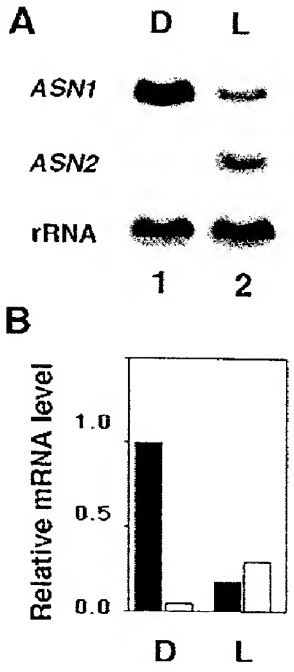


Figure 13. Relative levels of ASN1 and ASN2 mRNA in dark-adapted versus light-treated plants. Sixteen-day-old *Arabidopsis* seedlings were grown on tissue culture plates (MS plus 3% sucrose) and dark-adapted (D, lane 1) or light-treated (L, lane 2) for 48 h. Ten µg of total RNA was subjected to Northern blot analysis and hybridized with *ASN1* or *ASN2*. *ASN1* and *ASN2* gene-specific probes were approximately the same specific activity and X-ray exposure times were identical. 18S rRNA was detected on a replicate blot as a loading control. Following normalization to the corresponding rRNA, the highest level of mRNA (*ASN1*, lane 1) was set at 1.0. The bar graph **(b)** represents the relative levels of *ASN1* and *ASN2* transcripts in (a). From: Hon-Ming Lam, Ming-Hsiun Hsieh and Gloria Coruzzi (1998). Reciprocal regulation of distinct asparagine synthetase genes by light and metabolites in *Arabidopsis thaliana*. Plant Journal 16(3), 345-353.

various plant species (Lea and Miflin, 1980). Compared to glutamine, asparagine is relatively inert and carries more nitrogens per carbon. Thus, asparagine is used to store and transport nitrogen in many higher plant species including legumes, crop plants and also in *Arabidopsis*. While no higher plant mutants in AS exist to date, molecular and reverse genetic studies on ASN genes in *Arabidopsis* have begun to provide the first insights into the regulation of asparagine biosynthesis in plants.

Arabidopsis contains three genes for asparagine synthetase, ASN1, ASN2, and ASN3. ASN1 was cloned by homology to pea AS1 (Lam et al., 1994), while ASN2 and ASN3 were cloned functionally by complementation of a yeast asparagine auxotroph (Lam et al., 1998). The AS polypeptides encoded in each of these ASN cDNA clones contains a purF-type glutamine-binding domain (Richards and Schuster, 1992), suggesting that glutamine is the preferred substrate. Phylogenetic grouping suggests that ASN1 forms a separate clade from the ASN2 and ASN3 genes of *Arabidopsis*, suggesting the possibility that these enzymes may play distinct roles *in vivo* (Lam et al., 1998). The ASN2 & 3 genes group with monocot AS enzymes. Studies in maize suggest this form of AS encodes in form of the enzyme that uses Gln or ammonia as a substrate *in vitro* (Oaks and Ross, 1984).

The ASN genes also differ in expression patterns. ASN1 appears to be the major expressed gene in *Arabidopsis* seedlings (Lam et al., 1998). Expression of ASN3 is very low, and ASN2 expression while higher, is distinct from ASN1 suggesting that they serve distinct functions. ASN1 is expressed at highest levels in dark-adapted plants, and light inhibits its transcription (Fig. 13) and (Lam et al., 1994; Lam et al., 1998). By contrast, ASN2 is expressed at higher levels in the light (Fig. 13). The light repression of ASN1 and the light induction of ASN2 occur with 30 minutes and are kinetically reciprocal, suggesting distinct functions for the encoded enzymes (Lam et al., 1998). There are several pieces of evidence suggesting that ASN1 controls the major levels of free asparagine synthesized in *Arabidopsis*. 1. Levels of ASN1 mRNA parallel changes in levels of asparagine (Fig. 13), and 2. Transgenic manipulation of ASN1 (overexpression or antisense) affect levels of asparagine in plants (Lam et al., 2003)

Light also plays a major role in regulating asparagine levels in *Arabidopsis*, and this appears to be controlled at

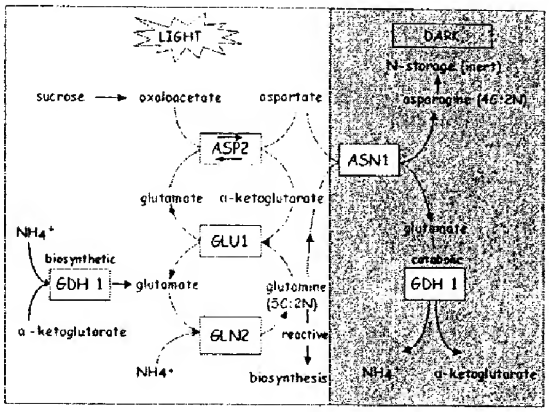


Figure 14. N-assimilation into Amino Acids is regulated by light. Cytosolic AAT2 controls the synthesis of aspartate in the light, which is converted to asparagine in the dark. A model is depicted for the metabolic flow of nitrogen assimilation into the nitrogen-transport amino acids glutamate, glutamine, aspartate, and asparagine in the light and dark. In the light, inorganic nitrogen is assimilated initially into glutamate and glutamine by the combined actions of the plastid enzymes: chloroplastic glutamine synthetase (GS2, encoded by *GLN2*), and ferredoxin-dependent glutamate synthase (Fd-GOGAT, encoded by *GLU1*; Oliveira *et al.* 1997; Coschigano *et al.* 1998). The conversion of glutamate into aspartate in the light is controlled by cytosolic AAT2. In the dark, this pool of aspartate is converted into asparagine by asparagine synthetase (*ASN1*) (Lam *et al.* 1994, 1995). From: Carolyn J. Schultz, Meier Hsu, Barbara Miesak and Gloria Coruzzi (1998). *Arabidopsis* Mutants Define an *in Vivo* Role for Isoenzymes of Aspartate Aminotransferase in Plant Nitrogen Assimilation. Genetics 149; 491-499.

the level of transcription of the ASN1 gene. Levels of asparagine are low in light grown plants, and high in dark adapted plants. Similarly, levels of ASN1 mRNA are low in light-grown plants and high in dark-adapted plants (Fig. 13). This light repression of ASN1 is mediated in part by phytochrome, and in part by light-induced changes in levels of photosynthate (Lam et al., 1994). More recently we have shown that blue light is also involved in repressing ASN1 expression (Thum et al., 2003). Studies have shown that sucrose (or glucose) can mimic the repressive effects of light on ASN1 expression when supplied to dark-adapted plants (Lam et al., 1998). Finally, why do plants use light and carbon to repress ASN1 transcription to affect asparagine biosynthesis? Because asparagine carries more nitrogens per carbon compared to glutamine, asparagine is used to transport nitrogen when levels of carbon are low. Hence, ASN1 is transcriptionally active in the dark, when levels of carbon are low, and transcription is repressed in the light, when carbon levels are high. Understanding the mechanisms by which light and carbon transcriptionally repress ASN1 is significant, because this transcriptional mechanism controls asparagine biosynthesis in *Arabidopsis*.

Light and Metabolic Control of Nitrogen Assimilation into amino acids

A significant amount of evidence has been accumulated to show that the genes regulating nitrogen assimilation into amino acids is subject to control at the transcriptional level. The signals affecting the transcription of N-assimilatory genes are light, carbon metabolites, and nitrogen metabolites. Below is a brief review of these three forms of regulation, as well as evidence for interactions between these signals. While effects of light on N-assimilation have been studied largely at the transcriptional level, they are also reflected at the level of amino acids. For example, the repression of ASN1 transcription by light (Lam et al., 1998), results in the specific accumulation of asparagine in dark-adapted plants (Lam et al., 1995). By contrast, Gln levels are higher in light-grown plants (Lam et al., 1995), and this is reflective of the induction of GLN2 transcription by light (Oliveira and Coruzzi, 1999). In *Arabidopsis*, the reciprocal control of GLN2 versus ASN1 by light at the mRNA level has been shown to reflect similar light-induced changes in the levels of glutamine and asparagine. Glutamine levels

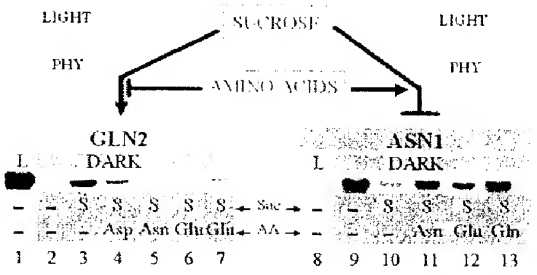


Figure 15. C:N Regulation of GLN2/ASN1 mRNA levels. Light and metabolites cause a reciprocal effect on *Arabidopsis* GLN2 and ASN1 gene expression. The mRNA levels of ASN1 are repressed to nearly undetectable levels in light-grown plants (lane 8) and are strongly enhanced in dark-adapted plants (lane 9). By contrast, the low, undetectable levels of GLN2 mRNA from plants grown in the dark (lane 2) are highly elevated in light-grown plants (lane 1). Sucrose can partially mimic the effects of light by causing the induction of GLN2 and repressing the expression of ASN1 mRNA in the dark (lane 10). In the dark, the light-mimicking effects of sucrose can be antagonized by treatment with amino acids (AA) (GLN2, lane 4 to 7 and ASN1, lanes 11 to 13) with each AA affecting the expression of GLN2 and ASN1 to different extents. The differential effects of the AA on both GLN2 and ASN1 mRNA levels may be explained by the possibility that each AA exerts its effects through different but partially overlapping pathways. However, one cannot rule out differences due to rate of uptake or metabolism. PHY, phytochrome; GLN2, *Arabidopsis* GS2 (glutamate synthetase) gene; ASN1, *Arabidopsis* gene AS1 (asparagine synthetase) gene. From: I.C. Oliveira, E. Brenner, J. Chiu, M.-H. Hsieh, A. Kouranov, H.-M. Lam, M.J.Shin and G. Coruzzi (2001). Metabolite and light regulation of metabolism in plants: lessons from the study of a single biochemical pathway. Brazillian Journal of Medical and Biological Research 34: 567-575.

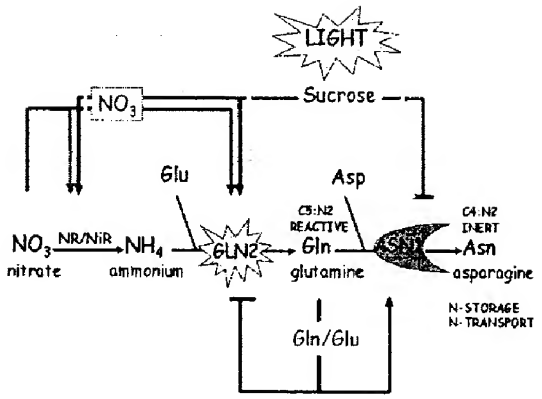


Figure 16. Multiple Input Regulation of N-assimilation genes. A simplified scheme depicting reciprocal regulation of GLN2 & ASN1 by Light, Carbon and Nitrogen. Also depicted are NR (nitrate reductase) and NiR (nitrite reductase).

are higher in light-grown plants, while asparagine levels are highest in dark-adapted plants (Schultz, 1994; Lam et al., 1995). Under light conditions, nitrogen is assimilated into metabolically active glutamine and glutamate, and transported as such for use in anabolic reactions in plants. Under dark conditions (low carbon concentration relative to organic nitrogen), the plants direct the assimilated nitrogen into inert asparagine for long distance transport or long-term storage (Fig. 14).

The light control of N-assimilatory genes such as ASN1 and GLN2 in *Arabidopsis* has been shown to operate via the plant photoreceptor phytochrome (Lam et al., 1994; Oliveira and Coruzzi, 1999) and by blue light (Thum et al., 2003). It appears that light also exerts an effect on the expression of other genes in the N-assimilatory pathway. For example, both GLN2 and the GLU1 gene encoding Fd-GOGAT are both induced by light (Coschigano et al., 1998) (Fig. 14). As the GS/GOGAT cycle affects N-assimilation, the coregulation of these genes by light serves to coordinate their expression with the production of C-skeletons during photosynthesis. Conversely, light has been shown to repress genes encoding ASN1 and GDH (Lam et al., 1994; Turano and Fang, 1998) (Fig. 14).

This coordinate regulation by light of N-assimilatory genes appears not only to involve phytochrome and blue light photoreceptors, but also reflects a coordination of N-assimilation by carbon availability. It has been shown that a carbon source such as glucose or sucrose can at least partially mimic the effects of light. In the case of GLN2, sucrose supplied to dark-adapted plants can at least partially mimic the inductive effects of light (Fig. 15) (Oliveira and Coruzzi, 1999; Thum et al., 2003). By contrast, the light repression of ASN1, can be at least partially mimicked by supplying sucrose to dark adapted plants (Fig. 15) (Lam et al., 1994; Thum et al., 2003). Studies on glucose control of gene expression in *Arabidopsis* suggest that the regulation of N-assimilation by carbon is mediated via a non hexokinase dependent pathway (Sheen et al., 1999).

This regulation of nitrogen assimilatory genes by the cellular carbon status appears to coincide with the interrelationship between carbon and nitrogen metabolism in plants. On the one hand, carbon assimilation and nitrogen assimilation compete for reducing power and energy sources generated by photosynthesis. On the other hand, carbon metabolism provides the necessary carbon backbones for the biosynthetic process of nitrogen assimilation while the products, e.g. amino acids, are essential components of the photosynthetic apparatus. In addition to the control by carbon status in the cell, it has been proposed that the relative abundance of nitrogen pools also plays a significant role in regulating nitrogen assimilation (Coruzzi and Bush, 2001; Coruzzi and Zhou, 2001). In fact, some reports claim that the cellular carbon to nitrogen ratio is a major player in the metabolic control of nitrogen assimilation (Coruzzi and Zhou, 2001; Foyer et al., 2003). Plant glutamate receptors uncovered by (Lam et al., 1998) have also been implicated in sensing changes in Glu and in C/N levels (Kang and Turano, 2003). In the case of N-assimilation gene expression, the notion that plants sense C:N ratio is supported by the data which shows that the carbon induction of GLN2 is reversed by the addition of organic N, while the carbon repression of ASN1 is relieved by the addition of organic N (Lam et al., 1994; Oliveira and Coruzzi, 1999) (Fig. 15). In more recent gene chip experiments, Crawford's group showed that nitrate treatments affected the transcription of a host of genes involved in nitrogen assimilation including nitrate and nitrite reduction, as well as genes involved in N-assimilation such as Fd-GOGAT and asparagine syntetase (Wang et al., 2000). Data using *Arabidopsis* mutants or GS inhibitors suggest that either inorganic N (nitrate) (Crawford and Glass, 1998; Stitt, 1999) and organic N (Gln) (Rawat et al., 1999) may serve as signals to report on N-status and control the expression of genes involved in nitrogen uptake (e.g. the ammonium transporter AMT1) and/or assimilation.

In addition to its regulation by factors such as light, carbon and nitrogen, the pathway of N-assimilation genes such as ASN1 is further affected transcriptionally by other forms of regulation including regulation by circadian control (Harmer et al., 2001). Thus, the control of N-assimilation in plants is subject to complex control mechanisms. Both forward and reverse genetic approaches have been initiated in *Arabidopsis* in order to uncover the molecular mechanisms by which plants regulate gene expression in response to C and N metabolites. These studies are in their infancy, and this section is prime for expansion in the subsequent updates of this chapter.

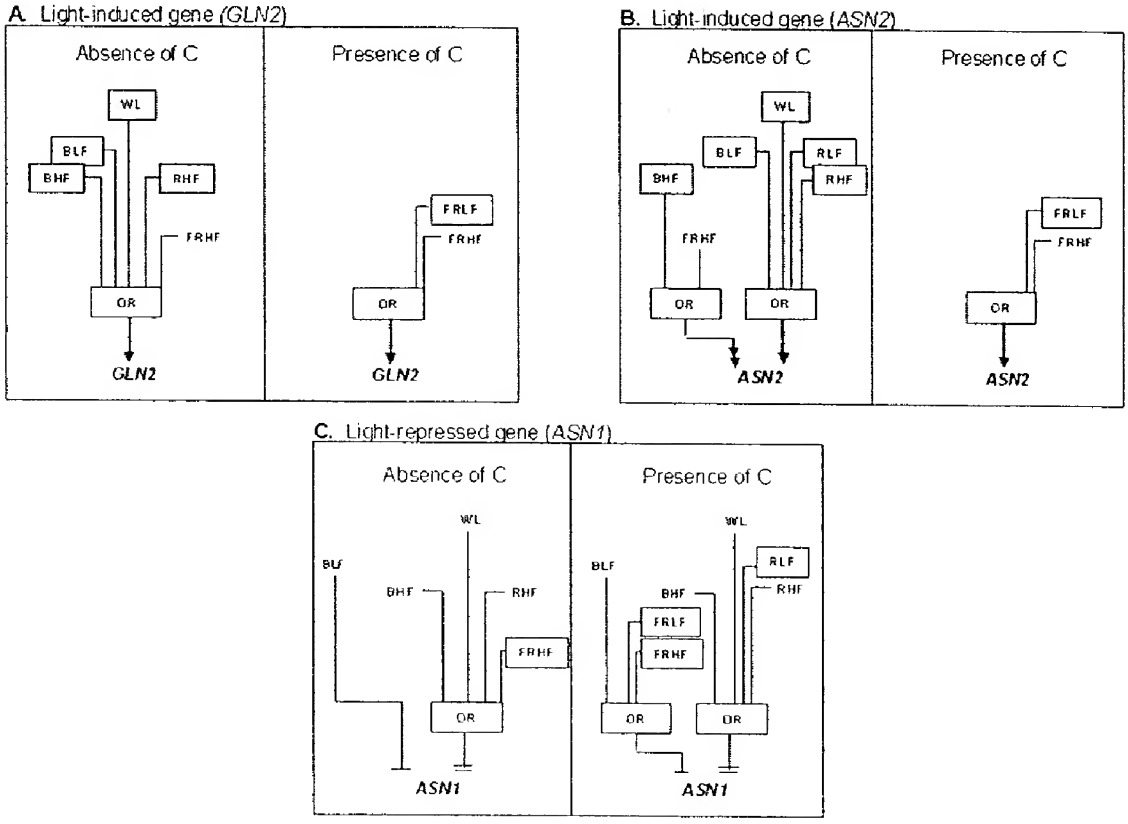


Figure 17. Boolean Circuits for Interaction of Light and Carbon (light grown). Boolean circuits model *ASN1*, *ASN2* and *GLN2* regulation by light and carbon in light-grown plants. A, B, and C Boolean circuits based on 16 experiments. **A.** *GLN2* regulation by white, blue, red or far-red light when compared against a base-case of no light, no carbon or no light, carbon. **B.** *ASN2* regulation by white, blue, red or far-red light when compared against a base-case of no light, no carbon or no light, carbon. **C.** *ASN1* regulation by white, blue, red or far-red light when compared against a base-case of no light, no carbon or no light, carbon. The inputs are white light (WL), blue light low fluence (BLF), blue light high fluence (BHF), red light low fluence (RLF), red light high fluence (RHF), far-red light low fluence (FRLF), far-red light high fluence (FRHF). Low fluence is 2 μmol m^{-2} s^{-1}, high fluence is 100 μmol m^{-2} s^{-1}. The arrow or barred lines indicate the function of the inputs as either inductive or repressive. Double arrows or double bars denote super-induction or super-repression, respectively. For a Boolean OR, if any one of the inputs is active, the output will also be active. Differences in the input for Boolean circuits when comparing 'absence of carbon' to 'presence of carbon' are shown as boxed inputs. From: Thum KE, Shasha DE, Lejay L, Coruzzi G (2003) Light- and Carbon Signaling Pathways. Modeling Circuits of Interactions. Plant Physiology 132: 440-452.

PERSPECTIVES

Our lab has recently begun to use a systems approach to model and integrate the intersection of multiple signals that regulate the expression of N-assimilatory genes (Shasha et al., 2001; Thum et al., 2003). In one approach, we have employed a math tool called Combinatorial Design to design a parsimonious set of experiments that cover all combinations of treatments with light, carbon, and nitrogen sources (Shasha et al., 2001). By covering such experimental spaces, we have been able to use Boolean logic to model how signals interact. In another recent study, we covered an experimental space that allowed us to model how light sources (red, blue, far red, white) interact with C sources (sucrose) in the regulation of genes in the N-assimilation pathway (Thum et al., 2003) (Fig. 17). Modeling the regulation of this pathway and incorporating metabolic data, is a first step to the creating of a virtual plant which may be used in a predictive mode to allow changes that will enhance N-assimilation in plants.

Molecular and genetic analyses of N-assimilation have provided important tools to extend our knowledge of nitrogen assimilation that was originally based on biochemical studies. The cloned genes and *Arabidopsis* mutants in specific genes have helped to distinguish the physiological roles of specific nitrogen assimilatory isoenzyme. The mechanisms by which light and/or

metabolic status (carbon and nitrogen) regulate nitrogen assimilation is beginning to be dissected using cloned genes and using genetic approaches. For example, some potential candidate regulatory genes have already been identified. In addition, specific screens for mutants in this process can be conducted in a genetic tractable system such as *Arabidopsis*. A combined molecular and genetic study of the regulatory network by which a gene responds to the metabolic status will lead to a better understanding of the genetic cross-talk between different carbon and nitrogen metabolic pathways. Basic research studies in these areas in *Arabidopsis* may also make significant contributions that can be applied to the improvement of nitrogen usage efficiency and crop yield in less genetically tractable systems.

ACKNOWLEDGMENTS

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Exhibit 3

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Lysine and threonine metabolism are subject to complex patterns of regulation in *Arabidopsis*

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Abstract

To study the regulation of lysine and threonine metabolism in plants, we have transformed *Arabidopsis thaliana* with chimeric genes encoding the two bacterial enzymes dihydrodipicolinate synthase (DHPS) and aspartate kinase (AK). These bacterial enzymes are much less sensitive to feedback inhibition by lysine and threonine than their plant counterparts. Transgenic plants expressing the bacterial DHPS overproduced lysine, but lysine levels were quite variable within and between transgenic genotypes and there was no direct correlation between the levels of free lysine and the activity of DHPS. The most lysine-overproducing plants also exhibited abnormal phenotypes. However, these phenotypes were detected only at early stages of plant growth, while at later stages, new buds emerged that looked completely normal and set seeds. Wild-type plants exhibited relatively high levels of free threonine, suggesting that in *Arabidopsis* AK regulation may be more relaxed than in other plants. This was also supported by the fact that expression of the bacterial AK did not cause any dramatic elevation in this amino acid. Yet, the relaxed regulation of threonine synthesis in *Arabidopsis* was not simply due to a reduced sensitivity of the endogenous AK to feedback inhibition by lysine and threonine because growth of wild-type plants, but not of transgenic plants expressing the bacterial AK, was arrested in media containing these two amino acids. The present results, combined with previous studies from our laboratory, suggest that the regulation of lysine and threonine metabolism is highly variable among plant species and is subject to complex biochemical, physiological and environmental controls. The suitability of these transgenic *Arabidopsis* plants for molecular and genetic dissection of lysine and threonine metabolism is also discussed.

Introduction

In plants, the essential amino acids lysine and threonine are synthesized from aspartate by two separate branches of the aspartate family pathway [5, 8]. This pathway is regulated by several feedback inhibition loops. Aspartate kinase (AK), the first enzyme of the pathway, is common to all amino acid end-products of the pathway and consists of several isozymes that are feedback-inhibited by lysine and threonine [5, 7, 8]. In addition, lysine inhibits the activity of dihydrodipicolinate synthase (DHPS), the first enzyme that is specific to lysine synthesis, while threonine feedback inhibits the activity of homoserine dehydrogenase, the first enzyme that is specific to the branch leading to threonine synthesis [5, 7, 8]. Analysis of various plant mutants possessing modified, feedback-insensitive enzymes have shown that DHPS plays a major regulatory role in lysine synthesis, while AK rate limits the synthesis of threonine [7, 8 and references therein]. This has also been confirmed by analysis of transgenic tobacco and potato plants expressing either hectopically or in a seed-specific manner feedback-insensitive AK and DHPS derived from *Escherichia coli* [15, 16, 20–23]. Notwithstanding, despite of similar expression levels of the bacterial transgenes in the two plant species, there were major differences between them in the extent and pattern of free lysine and threonine accumulation. For instance, in tobacco plants, free lysine level was increased sig-

nificantly in young shoots and was even more dramatically elevated in young leaves of more mature plants. In transgenic potato plants, free lysine levels were considerably lower than in transgenic tobacco and exhibited relatively little variability in various organs and stages of plant development. Moreover, in the tobacco plants, lysine overproduction was associated with substantially abnormal phenotypes while the lysine-overproducing potato plants looked entirely normal [20, 21, 23].

Analyses of the transgenic plants expressing the bacterial AK and DHPS genes suggested that lysine and threonine synthesis in plants is regulated not only at the biochemical levels, but apparently also by additional unknown factors that vary among plant species [7]. Thus, for further molecular and genetic dissection of these regulatory patterns, we have transformed the same bacterial genes into the model plant *Arabidopsis*. Interestingly, although DHPS and AK represent limiting factors for lysine and threonine synthesis also in *Arabidopsis*, our results showed striking differences between this plant and tobacco or potato in the extent and pattern of lysine and threonine accumulation as well as in the pattern and severity of the abnormal phenotypes.

Materials and methods

Chimeric genes and transformation vectors

The chimeric genes encoding the bacterial feedback-insensitive DHPS and AK used in this study have been previously described [21, 22]. These chimeric genes also included: (1) the 35S promoter of cauliflower mosaic virus; (2) a DNA encoding a pea plastid transit peptide from a pea *rbcS-3A* gene [6] to direct the bacterial enzymes into the organelle where the aspartate family pathway functions; (3) an Ω translation enhancer from the coat protein gene of tobacco mosaic virus [9]; and (4) a transcription termination and polyadenylation signal of the *Agrobacterium tumefaciens* octopine synthase gene [10]. The derivatives of the Ti plasmid pGA492 [1] harboring these chimeric genes were introduced into the *A. tumefaciens* strains EHA101 [12] and LBA4404 [11] by electroporation.

Plant transformation and growth

Arabidopsis ecotypes C24 and Columbia were transformed with the *A. tumefaciens* strains containing the chimeric DHPS and AK genes by the root transformation protocol [18]. Transformed, kanamycin-resistant *Arabidopsis* plants (T_0) were grown either in Magenta boxes containing GM (0.64% agar, 1× MS salts, 1% sucrose, 100 mg/l inositol, 1 mg/l thiamine, 0.5 mg/l pyridoxine, 0.5 mg/l nicotinic acid and 0.5 g/l MES buffer, pH 5.7) and 50 mg/l kanamycin, or in peat pellets (Jiffy 7) in growth room (25 °C, 16 h of cool white fluorescent light of 60 μE m^{-2} s^{-1} and 8 h dark) until maturity.

T_1, T_2 and T_3 plants were grown to maturity in a growth chamber with peat pellets as soil medium. Growth conditions were: 23 °C, 16 h of cool white fluorescent light of 100 μE m^{-2} s^{-1}; 8 h dark and 60–70% humidity. Plants were under irrigated.

Plant material for biochemical analyses

Plants were grown for 5–7 weeks in the growth chamber until large rosettes were obtained. Whole plants were then harvested and ground with an electric homogenizer in liquid nitrogen. Each sample was divided into two parts, one designated for free amino acid composition analysis and the second either for either DHPS activity or for western blot analysis. Frozen extracts were stored at −70 °C until used.

Analysis of DHPS and AK activities

A sample of 100 to 400 mg of frozen tissue was homogenized by an electric homogenizer in an equal volume of either cold 100 mM Tris-HCl buffer pH 7.5, containing 2 mM EDTA, 1.4% (w/v) sodium ascorbate, 1 mM phenylmethylsulfonylfluoride (PMSF), and 1 μg/ml leupeptin (DHPS activity) or 20 mM potassium phosphate buffer pH 7.0 containing 30 mM 2-mercaptoethanol, 0.1 mM L-lysine, 0.1 mM L-threonine, 1 mM PMSF and 0.5 μg/ml leupeptin (AK activity). After 15 min centrifugation (16 000×g, 4 °C), the supernatant was recentrifuged under the same conditions. The supernatant was collected and its protein concentration was determined by the method of Bradford [4]. Equal amounts of protein were then tested either for DHPS activity using the *O*-aminobenzaldehyde method [24], or for AK activity according to the method of Black and Wright [3]. One unit of DHPS activity was defined as the amount of activity necessary to produce a change in A_{540} of 0.001/min per mg protein [19].

Analysis of free amino acids composition in whole transgenic plants

Free amino acids were extracted from a sample of about 100 mg frozen tissue, as described previously [2]. Tissue was homogenized by an homogenizer in an 2 ml eppendorf tube in the presence of cold 500 μl double-distilled water (DDW):chloroform:methanol (3:5:15, v/v). After a short homogenization, 500 μl mixture were added and the sample was further homogenized until a homogenous texture was obtained. Tubes were then transferred to ice. Samples were centrifuged for 5 min (16 000$\times g$, 4 °C) and the supernatant was transferred to a new 2 ml tube. Chloroform (376 μl) and DDW (250 μl) were added to each sample, which was vortexed well and centrifuged for 5 min, (16 000$\times g$, 4 °C). The upper phase was collected, dried and dissolved in 200 μl DDW. Acetonitrile (0.3 ml) was added to each sample, and after centrifugation (30 min, 16 000$\times g$, 4 °C) the supernatant was collected, dried and dissolved in 150 μl of DDW. Each sample was then transferred through mini reversed-phase columns (Sep-pak, C_{18} cartridge, Waters) before the concentration of free amino acids was determined using the *O*-phthaldehyde reagent and measuring the 335/447 nm fluorescence [13]. Amino acid composition was determined by loading a sample of about 5 nmol of total free amino acids on Hewlett Packard 'Amino Quant' Liquid Chromatograph.

Western blot analysis

Equal amount of proteins were separated on 10% polyacrylamide-SDS gels [17]. Proteins were then transferred from the gels onto a 0.2 nitrocellulose membrane, stained by Ponsau-S and reacted with anti *E. coli* DHPS or AK serum [21, 22], using the ECL kit (Amersham) as recommended by the manufacturer.

Statistical analysis

A regression significance F-test of the Microsoft Excel program was performed to compare the correlation between enzyme activity and amino acids levels between the control and the transgenic plants.

Results

Expression of the E. coli *DHPS in transgenic* Arabidopsis *plants*

To study the regulatory role of DHPS in the biosynthesis of aspartate-family amino acids in *Arabidopsis thaliana* plants, we have transformed the ecotypes C24 and Columbia with a chimeric gene encoding the *E. coli* DHPS under the control of the 35S promoter of cauliflower mosaic virus. A DNA encoding a plastid transit peptide was also used to direct the bacterial enzyme to the organelle. To test for the expression and function of the chimeric gene, proteins were extracted from whole, 5–7-week old individual plants of the selfed T_1 and T_2 generations. Out of 14 plants, each derived from an independent transformation event, 10 expressed detectable levels of the bacterial DHPS as determined by western blot analysis with anti bacterial DHPS antibodies (data not shown) [20, 21]. Next, we analyzed the activity of DHPS as well as the level of free lysine in whole plants, grown for about 5–7 weeks in the growth chamber. Since in general there were no significant differences in the results between the Columbia and the C24 ecotypes, the results with both of them will be demonstrated together. As shown in Fig. 1A, many of the transgenic plants possessed significantly higher levels of DHPS activity (up to several hundred-fold), compared to control untransformed plants. Moreover, as shown in Fig. 1B, free lysine level in many of the transgenic plants was significantly higher than in control plants. In several of the transgenic plants, free lysine became the most abundant amino acid amounting to over 50 mol% (Fig. 1B). Nevertheless, as can be deduced from comparison of panels A and B in Fig. 1, there was no correlation between DHPS activity and free lysine level among the various transgenic plants, suggesting that lysine metabolism in *Arabidopsis* is regulated not only by the activity of DHPS.

As lysine synthesis occurs by a different branch than that leading to the synthesis of threonine and methionine, we wished to test the effect of lysine overproduction on the metabolism of these amino acids. As shown in Fig. 2, lysine overproduction in the transgenic plants was associated with a sharp reduction in the level of free threonine. Free threonine averaged ca.8 mol% in wild-type plants and was sharply reduced to a level of ca. 2 mol% as the lysine level increased from ca. 0.5 to ca. 5 mol%. No further reduction in free threonine level was evident with further increase in lysine level

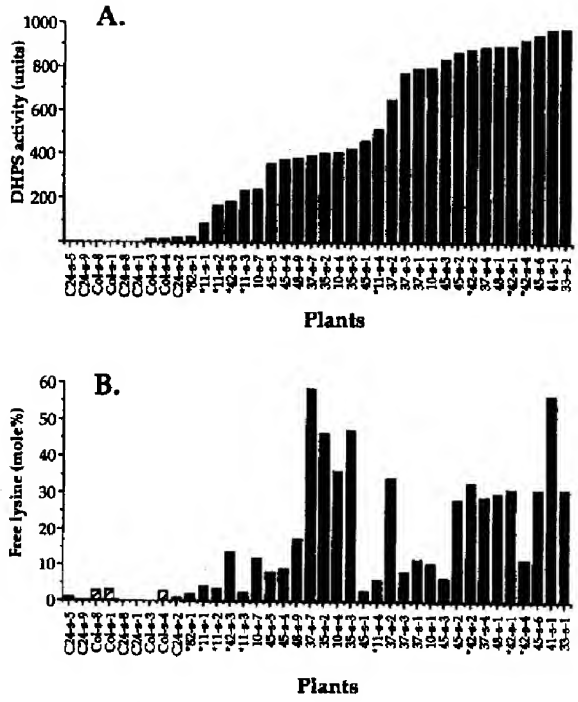

Figure 1. DHPS activity and free lysine levels in the transgenic plants expressing the *E. coli* DHPS. Whole 5–7-week old *Arabidopsis* plants were ground in liquid nitrogen, divided into two parts and analyzed for DHPS activity in crude extracts (A) and for free lysine levels (B). One unit of DHPS activity was defined as the amount of activity necessary to produce a change in A_{540} of 0.001/min per mg protein. C24-s-1 to C24-s-9, C24 control untransformed plants; Col-s-1 to Col-s-8, Columbia control untransformed plants; 82-s-1 to 33-s-1, transgenic plants expressing the *E. coli* DHPS. Columbia ecotypes are marked by an asterisk. Each column represents the results from a different individual plant, while columns carrying the same name represent different individuals of the same transformation event.

(Fig. 2). Methionine level was below the detection level in both the control and transgenic plants and therefore could not be studied.

We also studied the effect of lysine overproduction on the levels of other free amino acids beside threonine. Although no significant difference was obtained in any of the amino acids, the level of free aspartate, the precursor of the aspartate pathway, was slightly reduced in some of the transgenic plants (data not shown). Thus, although our results are in accord with previous data showing that aspartate levels are not limited in tobacco and potato [20, 21, 23], they suggest that production of aspartate may be slightly more limited in *Arabidopsis*.

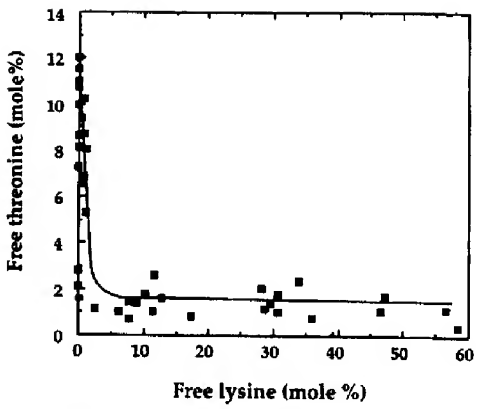

Figure 2. Correlation between free threonine and free lysine levels in the transgenic plants expressing the *E. coli* DHPS. Free amino acids were measured in whole 5–7-week old *Arabidopsis* plants.

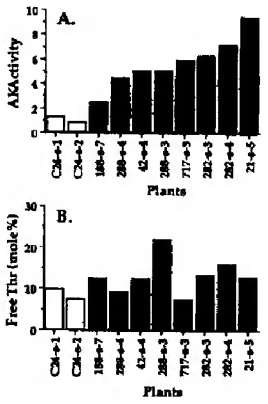

Figure 3. AK activity and free threonine levels in transgenic plants expressing the *E. coli* AK. Whole 5–7-week old *Arabidopsis* plants were ground in liquid nitrogen, divided into two parts and analyzed for AK activity in crude extracts (A) and for free threonine levels (B). C24-s-1 and C24-s-2, C24 control untransformed plants; 21-s-5 to 288-s-4, transgenic plants. Each column represents different individual plant examined while columns carrying the same name represent different individuals from the same transformation event.

Expression of the E. coli *feedback-insensitive AK in transgenic* Arabidopsis *plants*

The results shown in Fig. 2 were significantly different than those obtained in transgenic tobacco plants expressing the same chimeric gene. In the transgenic tobacco plants, free threonine level was ca. 2 mol% in wild-type plants and its level was not reduced with increasing lysine levels [23]. Yet, when transgenic tobacco plants expressing a feedback-insensitive bacterial AK, which overproduced free threonine (up to ca. 8 mol%) were crossed with the transgenic plants

expressing the bacterial DHPS [23], the increased lysine accumulation was significantly correlated with a reduction in threonine overproduction (reduction from ca. 8 mol% to ca. 2 mol%). Thus, our results suggested that in *Arabidopsis*, AK provides a less stringent control on threonine synthesis than in tobacco. To address this issue, we have also transformed wild-type *Arabidopsis* with the chimeric gene encoding the bacterial feedback-insensitive AK, driven by the 35S promoter [22]. To test for expression of the chimeric AK gene, proteins were extracted from whole, 5–7-week old individual plants of the selfed T_1 and T_2 generations. Western blot analysis with anti bacterial AK antibodies showed that out of 14 independent transformants, 11 exhibited detectable although variable levels of the bacterial AK (data not shown). We then tested the effect of expression of the bacterial AK of total AK activity and free threonine levels in several transgenic plants. As shown in Fig. 3A, B, although expression of the bacterial AK resulted in a notable increase of up to ca. 9-fold in AK activity, it generally did not cause any substantial elevation in free threonine levels. Analysis of threonine levels in a large number of transgenic plants (data not shown) indicated that except for four individuals which exhibited about 2-fold increase in the level of this amino acid (ca. 15–20 mol% threonine), all others were comparable to the control untransformed plants.

One possible way to interpret the results shown in Fig. 3 is that in *Arabidopsis*, the activity of the AK isozymes is not as sensitive to feedback inhibition by either lysine or threonine, compared to the respective AK activities in tobacco plants. To address this issue, wild-type and transgenic *Arabidopsis* plants, expressing the bacterial feedback-insensitive AK, were grown in medium containing lysine + threonine. The combination of these two amino acids generally retard plant growth because they completely inhibit the activity of all AK isozymes causing starvation for methionine [14]. As shown in Fig. 4, growth of wild-type *Arabidopsis* seedlings was significantly retarded on media containing 1 mM each of lysine + threonine, while that of the transgenic plants, expressing the bacterial AK was not. We have also studied the effect of lysine + threonine on growth of seedlings from the transgenic plants expressing the bacterial DHPS. In these plants, the endogenously overproduced lysine was expected to inhibit a significant portion of the lysine-sensitive AK. Indeed, as shown in Fig. 5, growth of these transgenic plants was significantly retarded on media containing 0.5 mM lysine + 0.75 mM threonine, while that of wild type was not.

Inheritance of the chimeric DHPS gene in the transgenic plants

To determine the number of insertions of the AK and DHPS transgenes in the transformed plants, T_1 progenies were tested for segregating genotypes carrying zero (control), one (heterozygous) and two (homozygous) doses of the transgenes. This was analyzed by germinating their selfed T_2 seeds on kanamycin-containing media. Since the yield of the T_1 seeds was very limiting, the number of T_1 plants that could be analyzed for each transformant was rather small. Nevertheless, in most cases analyzed, a segregation pattern of 1:2:1 of control: heterozygous: homozygous, respectively, was detected for kanamycin resistance. In several other plants, recessive individuals were found despite of the small number of plants examined. Heterozygous progenies of those plants further segregated at a 3:1 ratio upon selfing. This implied that in many of the transgenic plants , the chimeric gene was inserted into a single locus in the *Arabidopsis* genome.

Influence of the expression of DHPS on plant phenotype

Few of the transgenic plants, expressing the bacterial DHPS, exhibited altered phenotype (data not shown). The alterations were similar in general to the ones previously reported in transgenic tobacco plants, expressing the same chimeric gene [21, 23]. Yet, as opposed to the transgenic tobacco plants, the altered phenotypes of the *Arabidopsis* plants were very clear at relatively early stages of development, while at later stages they completely vanished leading to the formation of new normal-looking buds that developed fertile flowers and siliques.

Discussion

Regulation of lysine synthesis and its effect on plant phenotype in Arabidopsis

The results of this work show that like in many other plants, the synthesis of lysine in *Arabidopsis* is regulated at the biochemical level primarily by the sensitivity of DHPS to feedback inhibition by lysine. This was concluded from the observation that the transgenic

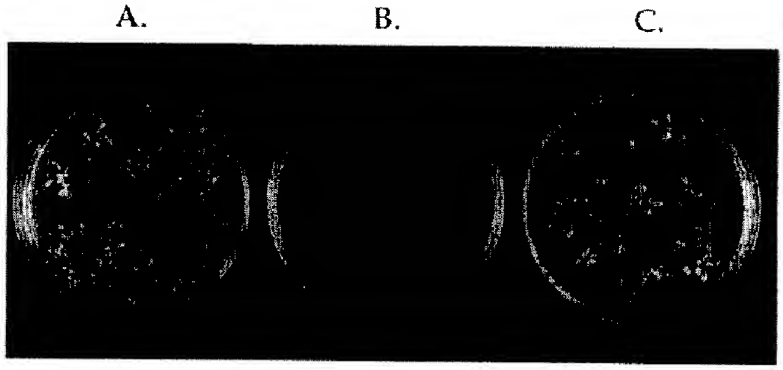

Figure 4. Sensitivity of the transgenic plants expressing the *E. coli* AK, to exogenously added lysine plus threonine. Seeds were germinated on medium supplemented with 1 mM lysine plus 1 mM threonine. Seedling development was monitored 3–4 weeks later. A. Untransformed C24 seedlings grown on germination medium lacking lysine plus threonine. B. Untransformed C24 seedlings on germination medium supplemented with lysine plus threonine. C. C24 transgenic seedling, line 282, on a germination medium supplemented with lysine plus threonine.

Arabidopsis plants, expressing the bacterial feedback-insensitive DHPS, accumulated higher levels of free lysine, compared to control untransformed plants. Moreover, we also found that the transgenic plants were also much more resistant than control plants to growth on media containing the toxic lysine analog *S*-aminoethyl L-cysteine (data not shown). Our results also showed that lysine overproduction in *Arabidopsis* was correlated with abnormal plant phenotype. Similar alteration in plant phenotype was previously reported in transgenic tobacco, but not in transgenic potato, expressing the same chimeric gene encoding the bacterial DHPS [20, 21, 23].

Although the sensitivity of DHPS to lysine inhibition generally plays an important role in lysine synthesis in plants, our results suggested that the metabolism of this amino acid is controlled by additional physiological and environmental factors, which vary between different plant species. This was concluded from the following observations: (1) in the transgenic *Arabidopsis* plants, as opposed to their counterpart tobacco and potato plants, there was no significant correlation between the activity of DHPS and free lysine levels; and (2) the abnormal plant phenotype, which results from lysine overproduction, was most prominent at early stages of *Arabidopsis* plant development and was vanished at later stages although the bacterial DHPS was expressed at high levels at all stages of plant development. Thus, the pattern of free lysine accumulation and plant phenotype in transgenic *Arabidopsis* plants are strikingly different than those obtained in two previously studied transgenic tobacco and potato plants expressing the same bacterial DHPS [20, 21, 23]. In transgenic tobacco, free lysine levels and the severity of the abnormal phenotypes increased with plant age [20]. In transgenic potato plants, free lysine levels were much lower than in tobacco throughout plant development and no sign of abnormal phenotype developed [20]. In transgenic *Arabidopsis* plants, the abnormal phenotype and apparently also the extent of lysine overproduction were highest at early stages of plant development and were significantly reduced toward later stages (this report). The physiological and environmental controls of lysine metabolism are currently being studied in our laboratory [7].

Regulation of threonine synthesis in Arabidopsis

Although AK has been shown to be the major limiting enzyme for threonine synthesis in many plant species [7], our results suggest that in *Arabidopsis*, the regulatory role of this enzyme is much more relaxed than in other plant species. This conclusion was deduced from the following: (1) free threonine level in wild-type *Arabidopsis* plants (ca. 8 mol%) was remarkably higher than in other plants, such as tobacco and potato (ca. 2.5 mol%); (2) expression of a bacterial feedback-insensitive AK had a considerably lower effect on threonine accumulation in *Arabidopsis*, than in tobacco [22]; and (3) in contrast to the situation in tobacco [23], overproduction of lysine in transgenic *Arabidopsis*, expressing the bacterial DHPS, was accompanied by a dramatic reduction in the threonine level. In fact, the negative correlation between lysine and threonine level, that was observed in the transgenic *Arabidopsis* expressing the bacterial DHPS, was

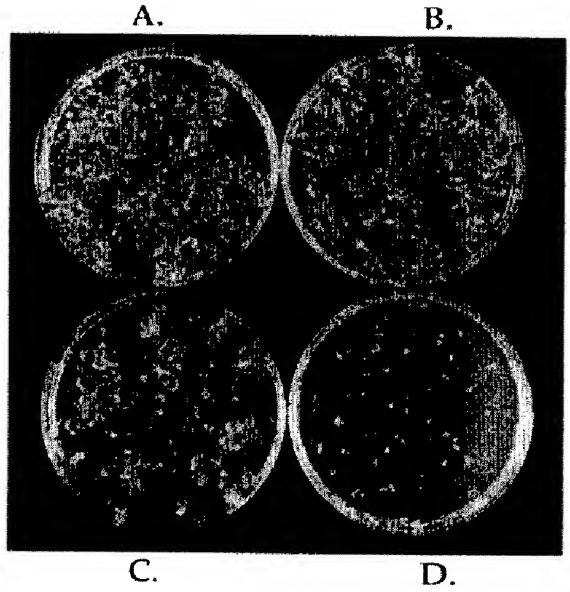

Figure 5. Sensitivity of the transgenic plants expressing the *E. coli* DHPS, to exogenously added threonine plus lysine. Seeds were germinated on medium supplemented with 0.75 mM threonine plus 0.5 mM lysine. Seedling development was monitored 3–4 weeks later. A C24 control untransformed plants grown on medium lacking threonine and lysine. B C24 transgenic plants, line 45, grown on medium lacking threonine and lysine. C C24 control plants grown of medium containing threonine plus lysine. D C24 transgenic plants, line 45, grown on medium containing threonine plus lysine.

also evident in transgenic tobacco plants expressing both the bacterial DHPS and feedback-insensitive AK [23]. This suggested that threonine production in wild-type *Arabidopsis*, was comparable to that in transgenic tobacco plants expressing the feedback-insensitive AK. The reason for the more relaxed regulation of threonine synthesis by AK in *Arabidopsis*, than in tobacco is yet unknown. However, this regulation is not due to the sensitivity of the various AKs to feedback inhibition by lysine and/or threonine, inasmuch as the sensitivity of both tobacco and *Arabidopsis* to growth on media containing lysine + threonine suggests that significant amounts of their AK activities are feedback-inhibited by these amino acids. Unraveling the regulation of threonine synthesis in *Arabidopsis*, is an issue of major current interest in our laboratory.

Arabidopsis *as a model plant for dissecting the regulation of lysine and threonine metabolism*

The results of this work suggest that the special patterns of regulation of lysine and threonine metabolism in *Arabidopsis*, compared to other plants, render this plant as an interesting model system to study these processes. Moreover, the availability of transgenic *Arabidopsis*, expressing AK and DHPS, that are much less sensitive to feedback inhibition by lysine and threonine than the endogenous enzymes, may open new avenues for the genetic, physiological and environmental dissection of lysine and threonine metabolism. The significantly higher sensitivity of the lysine overproducing transgenic *Arabidopsis* than wild-type plants to growth on threonine (in the presence of low concentrations of lysine) make it possible to select for mutants in which this sensitivity is suppressed. In addition, these transgenic plants may also be used for isolation of mutants with either suppressed or enhanced abnormal phenotypes. In addition, the transgenic *Arabidopsis* plants produced in this work may be crossed with a wide range of available *Arabidopsis* mutants, which are defective in various environmental or physiological factors, such as hormone action, in order to study the environmental and physiological controls of lysine and threonine metabolism.

Acknowledgements

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Patents

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Exhibit 4

Constitutive Overexpression of Cystathionine γ-Synthase in Arabidopsis Leads to Accumulation of Soluble Methionine and S-Methylmethionine[1]

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The committing step in Met and *S*-adenosyl-L-Met (SAM) synthesis is catalyzed by cystathionine γ-synthase (CGS). Transgenic Arabidopsis plants overexpressing CGS under control of the cauliflower mosaic virus 35S promoter show increased soluble Met and its metabolite *S*-methyl-Met, but only at specific stages of development. The highest level of Met and *S*-methyl-Met was observed in seedling tissues and in flowers, siliques, and roots of mature plants where they accumulate 8- to 20-fold above wild type, whereas the level in mature leaves and other tissues is no greater than wild type. CGS-overexpressing seedlings are resistant to ethionine, a toxic Met analog. With these properties the transgenic lines resemble *mto1*, an Arabidopsis, CGS-mutant inactivated in the autogenous control mechanism for Met-dependent down-regulation of CGS expression. However, wild-type CGS was overexpressed in the transgenic plants, indicating that autogenous control can be overcome by increasing the level of CGS mRNA through transcriptional control. Several of the transgenic lines show silencing of CGS resulting in deformed plants with a reduced capacity for reproductive growth. Exogenous feeding of Met to the most severely affected plants partially restores their growth. Similar morphological deformities are observed in plants cosuppressed for SAM synthetase, even though such plants accumulate 250-fold more soluble Met than wild type and they overexpress CGS. The results suggest that the abnormalities associated with CGS and SAM synthetase silencing are due in part to a reduced ability to produce SAM and that SAM may be a regulator of CGS expression.

Met is derived from Asp as are the amino acids Lys, Thr, and Ile. The committing step in Met synthesis occurs when the side chain of *O*-phosphohomoserine (OPH) condenses with the thiol group of Cys to form cystathionine (Fig. 1), an irreversible reaction catalyzed by CGS (EC 4.2.99.9). Cystathionine is cleaved to form homocysteine, which is then methylated with 5-methyltetrahydrofolate to form Met. The major metabolic fates of Met include its incorporation into protein, adenosylation to form SAM, and methylation to form *S*-methyl Met (SMM) (Fig. 1).

CGS competes with TS for OPH, their common substrate. Thus, TS may exert some control over the rate with which OPH is channeled toward Met (Bartlem et al., 2000; Fig. 1). TS is allostrically regulated by SAM (Curien et al., 1998) suggesting that Met synthesis could influence TS activity. Even so, several lines of evidence indicate that CGS controls the rate of Met synthesis. CGS activity decreases when Met is fed to the aquatic angiosperm *Lemna paucicostata* and increases when Met synthesis is blocked by inhibition of aspartokinase, the first enzyme in the biosynthesis of the Asp family of amino acids (Thompson et al., 1982). In the Arabidopsis mutant *mto1*, CGS is overexpressed, resulting in overaccumulation of soluble Met (Inaba et al., 1994; Chiba et al., 1999). Finally, antisense-RNA repression of CGS expression results in growth deformities stemming from an inability to synthesize Met (Gakiére et al., 2000; Kim and Leustek, 2000).

CGS expression may be regulated in Arabidopsis by an autogenous mechanism, revealed through analysis of the mutant *mto1* (Chiba et al., 1999). In wild-type Arabidopsis, Met or a metabolite thereof down-regulates CGS enzyme expression (Fig. 1) through a post-translational mechanism that acts by destabilizing CGS mRNA. In *mto1*, a point mutation in exon 1 of the CGS gene abolishes the Met-dependent destabilization of CGS mRNA causing the enzyme level and soluble Met level to rise. *mto1* was isolated by selection for Arabidopsis mutants that are resistant

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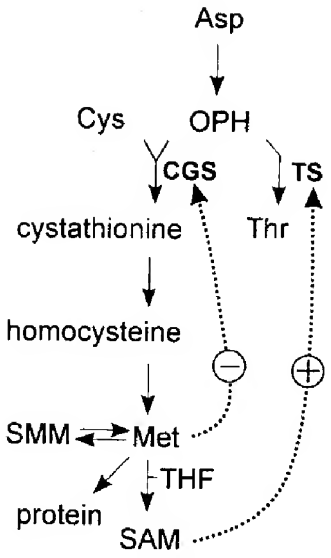

Figure 1. Pathway for synthesis of Met, SAM, and Thr. The pathway is as described in the text. SMM, *S*-methyl-Met; THF, 5-methyltetrahydrofolate; SAM, *S*-adenosyl-L-Met; CGS, cystathionine γ-synthase; TS, Thr synthase. Solid arrows refer to enzymatic steps and dotted arrows refer to regulatory steps. The arrow from Asp to OPH represents four enzymatic steps.

to ethionine, a toxic Met analog. Ethionine-resistance arises from overaccumulation of soluble Met.

In this study a transgenic approach was used to study the role that CGS protein abundance plays in controlling the level of free Met in Arabidopsis. The results show that transcriptional up-regulation of CGS causes accumulation of soluble Met and SMM, but only in specific tissues and stages of development. The results also show that transcriptional up-regulation of CGS can overcome the post-transcriptional mechanism controlling CGS expression. Cosuppression of CGS causes pronounced morphological aberrations and physiological changes that resemble those observed in Arabidopsis plants in which SAM synthetase (SAMS) is co-suppressed. Comparative analysis of CGS and SAMS-silenced plants suggests that SAM may be a regulator of CGS expression and that SAM deficiency may cause the morphological and physiological aberrations.

RESULTS

Isolation and Initial Characterization of Arabidopsis Lines with Altered Expression of CGS

Transgenic Arabidopsis plants were isolated from transformations with a construct intended to produce stable overexpression of CGS. A representative blot of leaf tissue from primary transformants analyzed for CGS protein level by immunoblotting shows that transgenic plants were isolated with a diversity of CGS levels in leaf tissue ranging from high-level expression (lines 31, 32, and 34) to plants from which CGS protein could not be detected (line 30; Fig. 2A). Reaction with an antibody against Ser acetyltransferase (SAT), an enzyme in the Cys biosynthesis pathway, illustrates that SAT, which is two biosynthetic steps before CGS, is unaffected in the transgenic plants. In total, 38 Kan-resistant plants were analyzed. Five showed high-level CGS expression (lines 14, 26, 31, 32, and 34) and many more showed an intermediate level of expression. In six of the primary transformants CGS protein could not be detected by immunoblotting (lines 16, 19, 20, 21, 23, and 30). The CGS-overexpression level was generally stable in the progeny of primary transformants up to the fourth generation, the last one that was analyzed. There were, however, exceptions that will be detailed later in this paper.

Three transgenic lines were chosen for further analysis (lines 14, 31, and 34), each from an independently transformed plant. The CGS construct was detected in each of the lines by PCR analysis (not

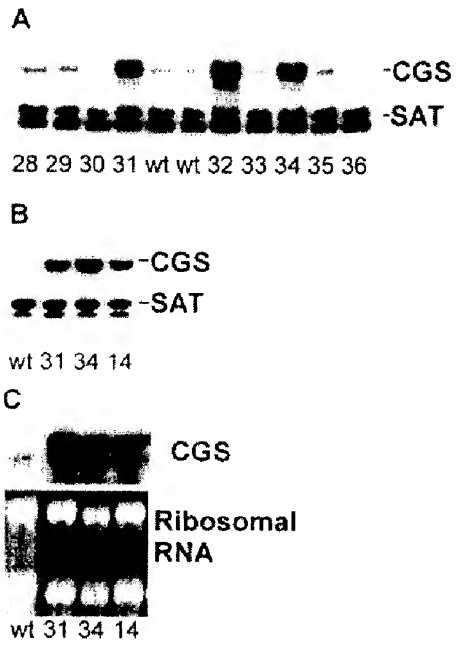

Figure 2. Blot analysis of CGS expression. A, Immunoblot of primary transgenic plants. The transgenic plants were selected on agar medium with 50 $\mu g\ mL^{-1}$ Kan for 7 d and then transferred to potting mix and grown for 30 d further. Ten micrograms of leaf protein was analyzed. B, Immunoblot of fourth generation homozygous CGS transgenic plants. The plants were grown in potting mix for 27 d. Ten micrograms of leaf protein was analyzed. C, RNA blot of fourth generation homozygous CGS transgenic plants grown as described in B. Ten micrograms of leaf RNA was analyzed for wild type and 5 μg for the transgenics. For all panels the transgenic plant tracking numbers or line names (wt, wild type) are indicated below the blots. Immunoblots were reacted at once with two antisera, one against CGS and the other against SAT. SAT serves as a protein loading control and it can be used in combination with the CGS antiserum because neither one interferes with the binding of the other to its specific antigen. CGS migrates at approximately 53 kD and SAT at approximately 39 kD. The band appearing below SAT is attributed to a reaction of the secondary antibody.

shown). RNA and immunoblot analysis showed that CGS is overexpressed in the leaves of 27-d-old plants (Fig. 2, B and C). Based on analysis of the intensity of the signal from the immunoblot CGS protein is 6- to 9-fold more abundant in the transgenic lines analyzed than in wild type. CGS enzyme activity measurements (CGS activity was measured with DPH as substrate) gave similar results. Lines 14, 31, and 34, respectively, showed 0.87 ± 0.06, 2.05 ± 0.07, and 1.10 ± 0.17 nmol min^{-1} mg^{-1} protein compared with an activity of 0.12 ± 0.04 for wild type. The intensity of hybridization signals from the RNA blot showed that CGS mRNA is 11- to 23-fold more abundant in the transgenics.

Plants That Overexpress CGS Are Resistant to Two Different Met-Pathway Toxins

Plants that overexpress CGS are phenotypically normal compared with wild type. The three lines chosen for detailed analysis can be visually distinguished from wild type by their resistance to ethionine and DL-propargylglycine (PAG). Ethionine resistance for two of them, lines 14 and 31 are shown in Figure 3A compared with *mto1*. Ethionine is a toxic Met analog and resistance can be overcome by overproduction of soluble Met (Inaba et al., 1994; Bartlem et al., 2000). This result indicates that CGS overexpression has probably resulted in Met overproduction in Arabidopsis seedlings.

PAG irreversibly inhibits CGS after binding to the enzyme active site (Johnston et al., 1979; Datko and Mudd, 1982; Ravanel et al., 1995). The specificity of this inhibitor is illustrated in Figure 3B. PAG at 60 μM inhibits the growth of wild-type Arabidopsis, however, growth is not inhibited if PAG is co-applied with 0.5 mM Met. All the CGS-overproducing lines showed resistance to PAG, as did the *mto1* mutant (not shown). Although PAG resistance in plants has never been reported before, it could theoretically arise from overproduction of active CGS, which would effectively titrate away the inhibitor by providing an excess of PAG binding sites.

Plants That Overexpress CGS Accumulate Free Met and SMM

Analysis of soluble amino acids revealed that Met and SMM accumulate in the CGS-overexpressing plants, but their level is strongly dependent on the developmental stage and organ (Fig. 4). The highest levels occur in the leaf and root of seedlings and in the root and flowers of mature plants. The accumulation of soluble SMM is more pronounced than is the accumulation of Met. The high accumulation of soluble Met and SMM in seedlings correlates with the resistance of seedlings to ethionine (Fig. 3A). As the CGS-overexpressing transgenic plants begin to flower, the level of Met and SMM declines in leaf

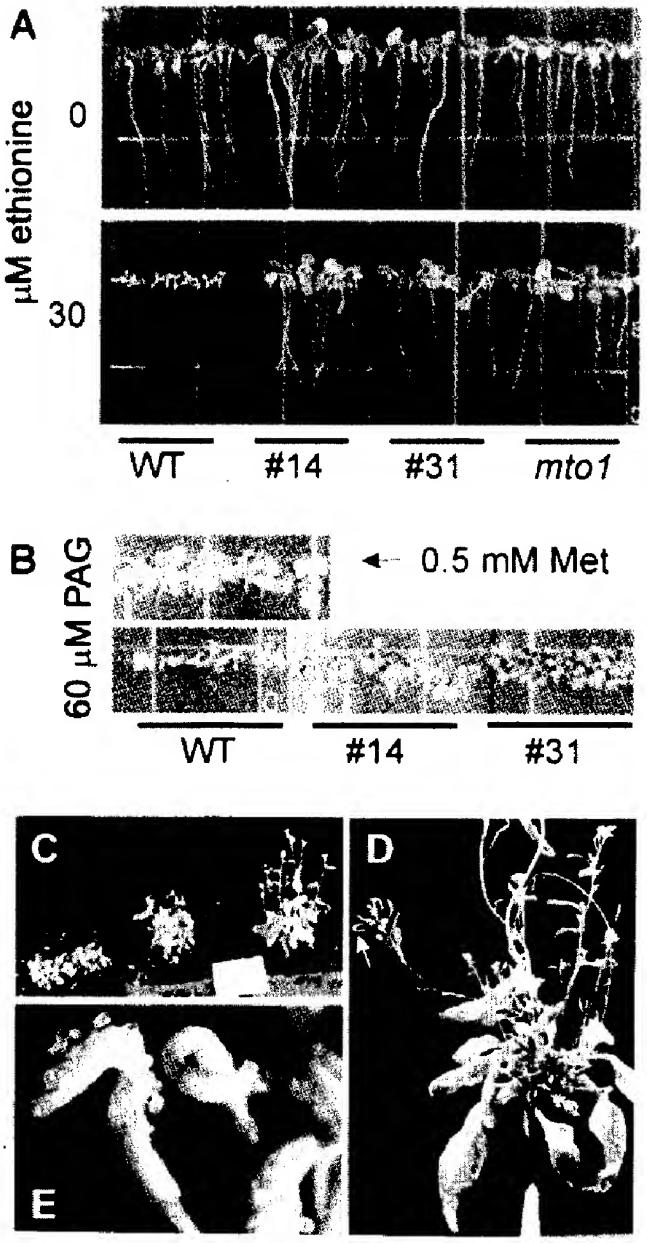

Figure 3. Resistance of CGS-overexpressing plants to ethionine or PAG, and phenotype of CGS-cosuppressed plants. A, Resistance to ethionine; B, resistance to PAG; C, response of plants that show CGS cosuppression early in development to exogenously applied Met; D, morphology of a plant in advanced stage of CGS cosuppression at the reproductive stage of development (the arrow indicates an example of a deformed silique); and E, close-up photograph of a deformed silique like that indicated by the arrow in D. Ethionine and PAG resistance were performed on fourth generation homozygous progeny of the indicated primary transgenic plants. The plants were grown on the indicated ethionine or PAG concentration. The reversal of PAG-induced growth inhibition by Met is shown in B. The plants shown in C are the third generation progeny from primary transformant 14 grown for 35 d in soil. The two plants in the pot on the left were not fed Met. The plants in the two pots on the right of the photograph were watered for 14 d with a nutrient solution containing 5 mM Met. The plant shown in D is a third-generation progeny of primary transgenic 14 grown for 45 d. The silique in E is from a third generation progeny of primary transformant 31 grown for 50 d.

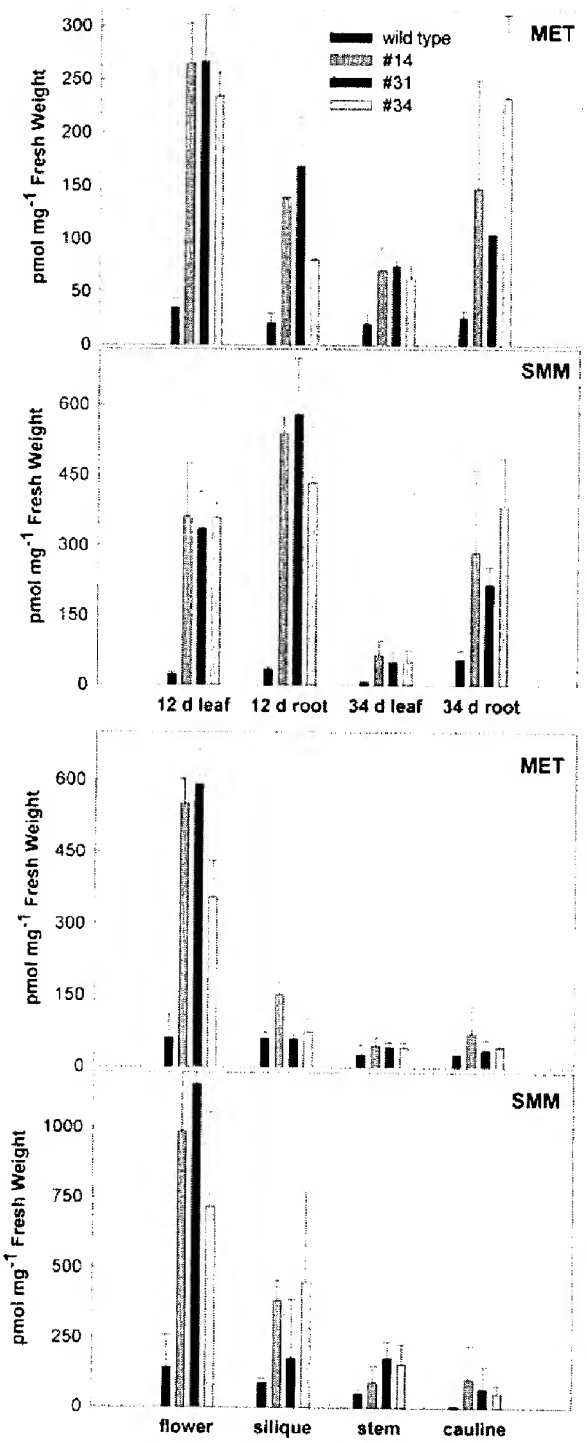

Figure 4. Soluble Met and SMM levels in tissues of wild-type and CGS-overexpressing plants. Plants of 12 d age were grown on agar medium. Plants of 34 d age were grown hydroponically. Flowers, siliques, stems, and cauline leaves were harvested from 38-d-old plants grown in potting mix. The identities of bars are as indicated in the inset of the top graph.

tissue. The level of these metabolites in roots is maintained, although the variation between plant samples increases. The level of Met and SMM in flowers is markedly greater than in wild-type flowers. In developing siliques, the inflorescence stem, and cauline leaves, Met and SMM are slightly elevated compared with wild type. As the plants progress through the flowering stage of development the level of Met and SMM in the leaf of transgenic plants declines further and is nearly indistinguishable from wild type, whereas the level remains high in flowers (not shown).

CGS Is Not Overproduced Equally in All the Organs of Transgenic Plants

Although it is expressed under the control of the 35S promoter, CGS is not uniformly overproduced throughout the plant. The immunoblot in Figure 5 shows it is strongly overproduced in young and older rosette leaves and in cauline leaves. Its level in stem, siliques, flowers, and roots (not shown) of the transgenic plants is far lower than in leaves. By comparison, SAMS is expressed to high levels in the stem and siliques, as has been previously reported (Peleman et al., 1989), and there is no difference in SAMS level between wild-type and CGS transgenic plants. Organ-specific CGS expression was similar in lines 14, 31, and 34, indicating that the expression pattern is a general feature of the transgenic plants. It is noteworthy that in flowering stage plants, the level of Met and SMM is inversely correlated with the level of CGS. The highest levels of Met and SMM occur in flowers and roots where CGS level is lowest, compared with leaves where the CGS level is high, but

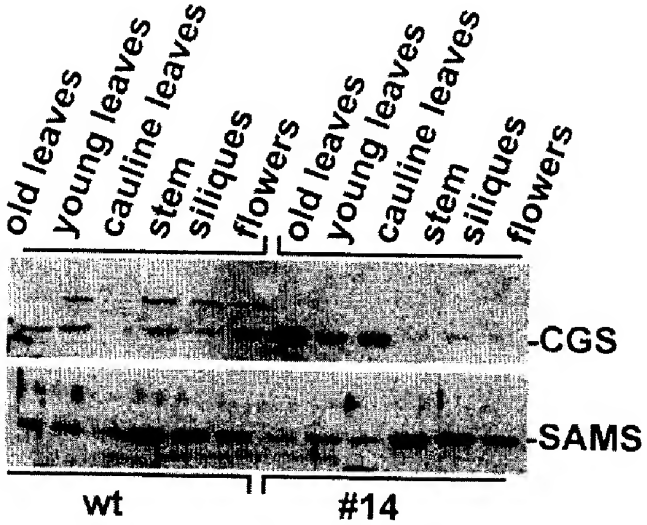

Figure 5. Immunoblot analysis of CGS and SAMS levels in various tissues from transgenic Arabidopsis that overexpress CGS. Plants from line 14 or wild type were grown for 45 d in potting mix. Immunoblotting was carried out on proteins from older, fully expanded leaves, young, immature leaves, cauline leaves, floral stem, siliques, and flowers harvested approximately at anthesis. The samples are described above and below the blots. Five micrograms of protein were analyzed for samples of wild type and 0.5 μg of samples from transgenic line 14.

Met and SMM are low. By contrast, in the tissues of young plants CGS level correlated positively with the level of Met and SMM (not shown). It is important to note that plants of normal morphology were used for the analysis of CGS expression described here.

Silencing of CGS Is Associated with Growth and Metabolic Abnormalities

Five of the primary transgenic plants derived from transformation with the construct intended for CGS overexpression did not show immunodetectable CGS protein (e.g. plant 30 in Fig. 2). The growth of these individuals was severely stunted and they were unable to produce inflorescences. The plants developed many, very small leaves, resulting from proliferation of numerous apical shoots, an indication that they may have reduced apical dominance. Application of Met as an addition to the watering solution stimulated vegetative and reproductive growth, although a completely normal morphology was not restored. However, with Met feeding, the plants were able to set viable seeds, which they could not without Met feeding. The seeds from Met-rescued plants in the second and later generations produced both normal, CGS-overexpressing, and abnormal, CGS-silenced progeny indicating that CGS silencing is unstable. The primary transgenic plants that initially showed CGS-overexpression also produced growth-stunted progeny that could be rescued by Met-feeding. A typical example from line 14 is shown in Figure 3C. Met was not applied to the two plants in the pot on the left, whereas Met was applied to the plants in the two pots on the right of the photograph. The growth of several inflorescences is visible in the Met-fed plants. The frequency with which severely stunted, CGS-silenced individuals were produced from a given line varied widely from one generation to the next and there was significant variation between transgenic lines. The simplest explanation for the phenotype of these plants is that silencing of CGS expression resulted in an inability to synthesize sufficient Met, or a metabolite thereof, for growth and that CGS silencing occurs spontaneously in response to epigenetic factors.

CGS cosuppression and associated morphological aberrations were also observed later in development. Such individuals were morphologically normal and overexpressed CGS until flowering had begun. Then, CGS silencing would occur in localized sectors, followed by a progressive advance of co-suppression throughout the plant and also in tissues that were previously normal. At an intermediate stage, it was possible to identify individual plants with both normal and abnormal organs. The immunoblot in Figure 6 shows that CGS is overexpressed in normal rosette leaves of such plants, but is cosuppressed in the abnormal rosette leaves. Silencing of CGS in such plants appeared to be spontaneous, occurring in only some progeny from a given line, and the frequency varied widely from one generation to the next and between transgenic lines. In some lines it was not at all observed.

A typical example of a plant at an advanced stage of CGS silencing is shown in Figure 3D. This particular individual grew normally and had produced a normal inflorescence at the time that the first symptoms of CGS silencing became evident. The normal inflorescence was cropped from the top of the photograph to focus on the aberrant morphology. At the time the photograph was taken all the leaves of this individual, which were initially of normal morphology, had became curled and developed small chlorotic patches. A second flush of abnormal inflorescences were produced, characterized by tightly clustered flowers and siliques, suggesting that apical dominance was lost and that elongation of the inflorescences had been curtailed. The abnormal development of siliques was particularly striking. During the early stages of growth the siliques split open, exposing the young ovules (Fig. 3E). The rupture would occur in the ovary wall, not at the dehiscence zone, and it appeared to arise from precocious enlargement of the ovules without a compensatory growth of the ovary wall. In other cases siliques would not split, but the enlarged ovules would produce a distinctive bumpy surface and curled structure as indicated by the arrow in the photograph of Figure 3D.

Table I. *Amino acid content in transgenic Arabidopsis that overexpress or cosuppress CGS*

Soluble amino acids were measured in tissues from 52-d-old plants. The samples from transgenic plants were taken from individuals showing CGS cosuppression (cs), and a plant from line 34 that overexpresses CGS (oe). The averages ± SD of three independent plants are shown with the exception of 34, which is a measurement from a single plant. For plant-to-plant variation for line 34, readers are referred to the data in Figure 4, which was obtained from 38-d-old plants. Differences between the value presented here for 34 and in Figure 4 may be due to differences in plant age. Total amino acid content represents the relative level calculated as the sum of all peak areas from chromatograms divided by the fresh weight of the plant sample. Amino acids were measured using the AQC method.

Sample	Amino Acid Content			Total Amino Acid Relative Value
	Met	SMM	Thr	
	pmol mg^{-1} fresh wt			
Leaf wild type	9 ± 4	0	407 ± 98	1.0 ± 0.3
Leaf #31cs	7 ± 7	0	2964 ± 1346	3.8 ± 0.7
Leaf #34cs	10 ± 2	0	1820 ± 382	3.0 ± 0.9
Leaf #34oe	27	0	365	1.2
Stem wild type	9 ± 7	5 ± 9	1298 ± 515	1.0 ± 0.3
Stem #31cs	11 ± 1	0	5018 ± 1655	1.6 ± 0.3
Stem #34cs	6 ± 4	0	7091 ± 3349	2.9 ± 0.3
Stem #34oe	43	15	1082	1.3
Silique wild type	10 ± 5	79 ± 29	1310 ± 365	1.0 ± 0.2
Silique #31cs	32 ± 37	80 ± 77	4272 ± 514	3.1 ± 0.8
Silique #34cs	67 ± 1	171 ± 54	10985 ± 792	3.9 ± 0.8
Silique #34oe	765	1592	1617	1.1

Table II. *Changes in amino acid content of CGS cosuppressed Arabidopsis*

The fold increase in amino acid content is given for leaf and silique from lines 31 and 34. The Asn and Ser peaks are not resolved on the chromatograph nor are the Gln and His peaks. Cys and Trp peaks could not be identified due to interference from unidentified compounds.

Fold Increase	Plant Line and Organ			
	31 Leaf	34 Leaf	31 Silique	34 Silique
1	Met	Met	Asn/Ser Asp Gln/His Glu	Asn/Ser Gln/His Glu
2 to 4	Gly	Ala Asp Gln/His Gly	Ala Arg Gly Leu Met Pro Thr Tyr Val	Ala Asp Gly Met
5 to 9	Ala Asn/Ser Asp Glu Leu Lys Phe Tyr Val	Asn/Ser Glu Ile Leu Lys Phe Thr Tyr Val	Ile	Arg Leu Phe Thr Val
10 to 20	Arg Gln/His Ile Pro Thr	Arg Pro	Lys Phe	Ile Lys Pro Tyr

Soluble amino acid analysis revealed extensive changes throughout plants in the advanced stages of CGS cosuppression. The levels of Met and SMM in the leaves and floral stem of normal plants is comparable with wild-type and CGS-overexpressing plants (Table I). However, Thr is 3.3- to 8.3-fold greater in the CGS-cosuppressed plants than in wild type, and total amino acids are up to 3.8-fold greater (Table I). The amino acids contributing to the overall increase in total amino acids are shown in Table II. From these results it is evident that a wide range of amino acids contribute to the increase.

The Morphology of CGS-Silenced Plants Resembles That Associated with SAMS Silencing

During the course of this study, it became evident that the morphology resulting from CGS silencing is remarkably similar to that of transgenic Arabidopsis cosuppressed for SAMS. Preliminary characterization of SAMS silencing in Arabidopsis has been reported by Boerjan (1993) and de Carvalho et al. (1994) and is in many respects similar to the effects of SAMS cosuppression in tobacco (Boerjan et al., 1994). Transgenic Arabidopsis lines showing cosuppression for CGS and SAMS were grown side by side for a more detailed comparison. The phenomena of CGS and SAMS cosuppression share the properties of being sporadic, hyper-variable, and localized to sectors on a single plant. The plants in which SAMS becomes cosuppressed early in development are severely stunted, produce numerous apical shoots, and are unable to flower. These are termed the MUT3 morphotype using the nomenclature of Boerjan (1993) and de Carvalho et al. (1994). MUT3 plants are nearly indistinguishable from plants in which CGS becomes cosuppressed early in development. A major difference is that the growth of SAMS cosuppressed plants cannot be restored by exogenous application of Met. Plants that develop SAMS cosuppression later in development produce curled, chlorotic leaves, and distorted siliques resulting from early enlargement of ovules, similar to plants where CGS becomes silenced later in development. These are termed the MUT2 morphotype (Boerjan, 1993; de Carvalho et al., 1994). Plants that overexpress SAMS are morphologically indistinguishable from wild type and are termed the MUT1 morphotype (Boerjan, 1993; de Carvalho et al., 1994).

Silencing of SAMS Causes CGS Overexpression and Accumulation of Met, SMM, and Other Amino Acids

In considering the significant morphological similarities associated with SAMS and CGS silencing it is noteworthy that these enzymes are metabolic neighbors in the pathway for SAM synthesis. It was, therefore, of interest to compare the physiological properties of SAMS-silenced plants with those that overexpress SAMS and wild type. Immunoblot analysis confirmed that the SAMS protein is overproduced in the leaves of the MUT1 morphotype and is reduced in the MUT2 and MUT3 morphotypes (Fig. 7), as has previously been shown by SAMS activity measurements (Boerjan, 1993; de Carvalho et al., 1994). In contrast, CGS is overexpressed in MUT2

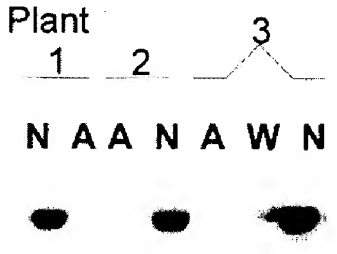

Figure 6. Immunoblot analysis of CGS level in leaf extracts from transgenic Arabidopsis plants showing normal (N) and abnormal (A) morphology compared with wild type (W). Abnormal is defined in the text. Plants from line 14 were grown in potting mix for 45 d. Five micrograms of protein was analyzed.

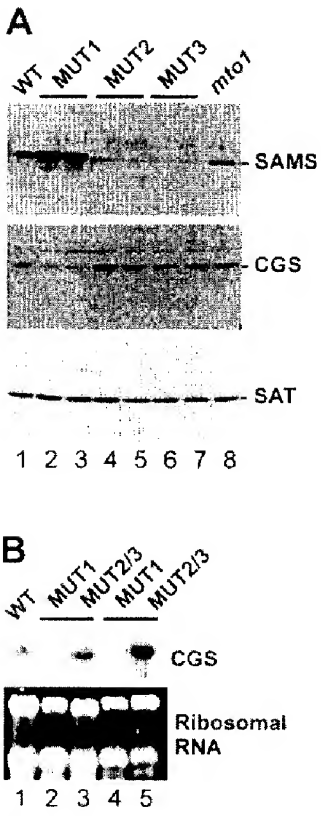

Figure 7. Blot analysis of leaf tissue from plants transformed with the SAMS construct. The plants were grown for 35 d in potting mix, and leaf samples from two individual plants of each morphotype were analyzed along with wild type and the *mto1* mutant. A. Immunoblots with the indicated antisera. B, RNA blot lane 2 and 3, line 27; lane 4 and 5, line 37. The samples are identified above the blots.

and MUT3, whereas its expression is slightly lower in MUT1 compared with wild type. Measurement of CGS enzyme activity in the leaves of MUT3 plants revealed that it is 0.91 ± 0.03 nmol min^{-1} mg^{-1} protein, compared with wild-type activity (0.33 ± 0.06), indicating that MUT3 plants have approximately 3-fold higher CGS activity than do wild-type plants. In contrast, SAT expression is unaffected.

Amino acid analysis revealed that the level of soluble Met and SMM are similar in wild type and in MUT1, but these metabolites accumulate to high levels in MUT2 and MUT3 (Table III). This property is in contrast to CGS-silenced plants, which do not accumulate Met and SMM. However, Thr and total amino acids are markedly increased (Table III). The amino acids contributing to the increase are listed in Table IV. The bulk of the increase is attributed to Arg, Pro, Met, and Lys, which are 145- to 568-fold more abundant than in wild-type or MUT1 plants.

DISCUSSION

The rationale for overexpressing CGS in transgenic Arabidopsis was to test whether this enzyme plays a role in regulation of Met synthesis, a hypothesis based upon both physiological (Thompson et al., 1982) and genetic (Chiba et al., 1999) evidence. The accumulation of soluble Met and SMM in the transgenic lines overexpressing CGS indicates that CGS is indeed a control point. The evidence from CGS transgenics, in combination with analysis of SAMS transgenic plants, produced insight into four additional points: (a) The observation that Met accumulation does not correlate with CGS level in a specific tissue indicates that the tissue concentration of soluble Met is controlled by factors other than the level of CGS activity; (b) the observation that CGS is not overproduced in some plant tissues, even though expression was transcriptionally deregulated from the transgene construct, suggests that a post-transcriptional mechanism may be involved in control of CGS expression; (c) the morphological similarity of CGS and SAMS-silenced plants suggests that the growth abnormalities are due in part to a reduced ability to produce SAM; and (d) the observation that CGS is overexpressed in SAMS-silenced plants provides indirect evidence that CGS expression may be controlled by SAM or some metabolite downstream of SAM. Each of these points will be individually addressed in the discussion that follows.

The leading hypothesis for the control of Met synthesis proposes that regulation occurs from the interplay between CGS and TS, two enzymes that compete for a common substrate, OPH (Giovanelli et al., 1980). In wild-type plants the level of Met and its metabolite SAM, control the partitioning of OPH. SAM activates TS (Curien et al., 1998), and Met or one of its metabolites represses CGS expression (Thompson et al., 1982; Chiba et al., 1999; Fig. 1). The finding reported here, that transcriptional up-regulation of CGS increases the soluble Met and SMM level in Arabidopsis, supports this hypothesis. Yet, CGS

Table III. *Amino acid content in transgenic Arabidopsis that overexpress or cosuppress SAMS*

Soluble amino acids were measured in the leaves of 44-d-old plants. The averages ± SD of three independent plants are shown. MUT1, -2, and -3 are morphotypes as described in the text derived from two different transgenic lines termed line 27 and line 37. Total amino acid content represents the relative level calculated as the sum of all peak areas from chromatograms divided by the fresh weight of the plant sample. n/d, Not determined. Amino acids were measured using the ninhydrin method. SMM was measured using the DMS release assay.

Sample	Amino Acid Content			Total Amino Acid Relative Value
	Met	SMM	Thr	
	pmol mg^{-1} fresh wt			
Wild type	12 ± 4	11 ± 2	702 ± 97	1.0 ± 0.2
MUT1 27	15 ± 5	5 ± <1	581 ± 78	1.1 ± 0.1
MUT1 37	17 ± 2	4 ± <1	783 ± 34	1.1 ± 0.2
MUT2 27	1,962 ± 78	n/d	2,265 ± 160	2.9 ± 0.1
MUT2 37	1,955 ± 106	n/d	2,130 ± 105	2.3 ± 0.1
MUT3 27	2,086 ± 287	424 ± 83	2,856 ± 376	4.0 ± 1.0
MUT3 37	2,967 ± 249	489 ± 48	3,343 ± 231	5.0 ± 1.0

Table IV. *Changes in amino acid content of SAMS cosuppressed Arabidopsis*

The fold increase in amino acid content is given for MUT2 and MUT3 morphotypes of SAMS lines 27 and 37. The Asn and Ser peaks are not resolved on the chromatograph nor are the Gln and His peaks. Cys and Trp peaks could not be identified due to interference from unidentified compounds.

Fold Increase	Plant Line and Morphotype			
	27 MUT2	27 MUT3	37 MUT2	37 MUT3
1	Ala Asp Glu Gly Phe Tyr		Asp Glu	
2 to 4	Asn/Ser Ile Leu Thr Val	Asp Glu	Ala Asn/Ser Tyr	Asp Glu Tyr
5 to 20	Arg Gln/His	Ala Asn/Ser Gly Phe Thr Tyr Val	Gln/His Gly Ile Leu Phe Thr Val	Ala Asn/Ser Gln/His Gly Leu Phe Thr Val
21 to 568	Lys Met Pro	Arg Gln/His Ile Leu Lys Met Pro	Arg Lys Met Pro	Arg Ile Lys Met Pro

overexpression did not cause Thr to decline in proportion to the increase in Met and SMM, as might have been expected if CGS and TS compete for a fixed pool of OPH. This result suggests that the OPH pool is not fixed and that the rate of OPH synthesis may change in response to increased OPH utilization by CGS.

In young transgenic Arabidopsis tissues the level of CGS protein correlates with accumulation of soluble Met and SMM, however, this relationship does not hold for older plants. The levels of Met and SMM decline in leaves at the time that flowers are produced (Fig. 4). Although CGS is much lower in flowers, Met and SMM accumulate in them. A similar phenomenon occurs in roots of reproductive stage plants, in which CGS expression is much lower than in leaves, yet roots accumulate Met and SMM (Fig. 4). Both of these findings suggest that in reproductive stage plants, overaccumulated Met and SMM could be transported into flowers and roots, rather than being synthesized in situ. The most likely source tissues could be rosette and cauline leaves where CGS is overproduced, but where Met and SMM do not accumulate. Transport of Met/SMM during flowering may be a general feature of Arabidopsis, because the simultaneous decrease in soluble Met in rosette leaves and increase in flowers has also been observed in the *mto1* and *mto2* Arabidopsis mutants (Inaba et al., 1994; Bartlem et al., 2000).

The notion that Met is redistributed in plants has recently been raised by the finding of Bourgis et al. (1999) that SMM is a major amino acid constituent in the phloem sap of a wide range of angiosperms. SMM and Met are interconverted through the action of SAM-dependent Met *S*-methyltransferase (EC 2.1.1.12) and SMM:homocysteine *S*-methyltransferase (EC 2.1.1.10). Together these enzymes form the SMM cycle. If localized within the same cell the cycle would be futile. However, spatial separation of these enzymes such that SMM synthesis predominates in leaves and SMM→Met reconversion occurs in sink tissues, provides a mechanism for redistribution of Met (Hanson et al., 2000).

Analysis of various organs of transgenic Arabidopsis revealed that CGS expression varies widely in different tissues (Fig. 5) even though expression is under control of the potent and constitutive cauliflower mosaic virus 35S promoter. Although this promoter is not equally active in all cells (Holtorf et al., 1995), tissue-specific differences in activity cannot account for the inability to overexpress CGS in the stem, flowers, and roots of the transgenic plants. Thus, the evidence points toward a post-transcriptional mechanism for regulation of CGS expression in Arabidopsis. Paradoxically, it is the flowers and roots of flowering-stage plants that accumulate Met and SMM (Fig. 4). A Met-dependent autogenous control mechanism has been identified for Arabidopsis CGS (Chiba et al., 1999) that centers on a small conserved region of amino acid residues encoded by exon 1 of the *CGS* gene termed the MTO1 domain. In response to elevated Met (or one of its metabolites, possibly SAM, as will be described below), the MTO1 domain mediates the destabilization of *CGS* mRNA. This model could explain why the level of CGS is inversely correlated with soluble Met/SMM level in flowers and roots of flowering stage CGS transgenic plants. Perhaps, autogenous control is avoided in rosette leaves by rapid export of Met or SMM, whereas in sink tissues where Met and SMM accumulate autogenous control represses CGS expression. The post-translational control hypothesis does not explain, however, why CGS level is not repressed in young Arabidopsis leaves and roots even though Met and SMM are overaccumulated. Autogenous control of CGS has been demonstrated in seedling tissues (Inaba et a., 1994; Chiba et al., 1999). Perhaps, the higher level of *CGS* mRNA derived from the transcriptionally deregulated transgene overcomes the autogenous control mechanism. A factor that complicates interpretation of the results of CGS expression in transgenic plants is the fact that CGS becomes silenced in a high proportion of transgenic lines. Thus, it is possible that low-level expression of CGS in certain organs may be the result of cosuppression rather than

autogenous regulation. However, this explanation cannot be generally true. Multiple independent samples were measured for each transgenic line, and for some lines the frequency of CGS silencing is low (<25% of the siblings), yet all showed a CGS expression pattern similar to the other lines. Moreover, care was taken to harvest samples only from plants with normal morphology in which, presumably, CGS silencing had not occurred.

The morphologies of CGS- and SAMS-silenced plants are remarkably similar, suggesting that the growth abnormalities are due in part to a reduced ability to produce SAM. Similar morphological aberrations are produced in Arabidopsis by inhibition of CGS expression through antisense RNA (Kim and Leustek, 2000). Both Met and SAM play central roles in plant metabolism. Thus, the question of which developmental processes are disrupted by the loss of CGS and SAM is complex. Indeed, it is more likely that many developmental processes are affected. For example, SAM is the precursor of ethylene and certain polyamines, and it serves as the methyl group donor in DNA methylation reactions. Ethylene and polyamines are known to play prominent roles in growth, flowering, and fruit development, so disruption of these processes by CGS and SAMS cosuppression is expected. Of potential significance is the phenotype of the Arabidopsis *acl5* mutant carrying a mutation in the spermine synthase gene, which shows growth stunting and produces siliques with a bumpy surface (Hanzawa et al., 1997; Hanzawa et al., 2000), morphologies reminiscent of CGS- and SAMS-silenced plants. Inhibition of DNA methylation produces defects in apical dominance and flowering (Finnegan et al., 1996; Ronemus et al., 1996), also characteristic of CGS- and SAMS-silenced plants. Finally, several groups have reported that CGS expression is increased in developing fruits (Nam et al., 1999; Hadfield et al., 2000; Marty et al., 2000), and SAMS is highly expressed in developing pea seeds (Gomez-Gomez and Carrasco, 1998), further indicating their likely importance in fruit development.

An interesting property of plants showing CGS or SAMS cosuppression is the large scale, global increase in amino acids. Amino acids also accumulate in Arabidopsis where CGS level is reduced by expression of antisense RNA (Kim and Leustek, 2000). The only major difference between the lines is that SAMS-silenced plants accumulate Met and SMM, whereas CGS-silenced plants do not. There likely are multiple reasons for the increase in amino acids. The first possibility is that a block in translation causes free amino acids to accumulate. The silencing of CGS results in a limitation of Met (Fig. 3C), which probably causes translation to stall, slowing the rate of amino acid incorporation into proteins. Similar global increases in amino acids occur when a variety of amino acid biosynthetic enzymes are blocked with inhibitors or through antisense RNA techniques (Guyer et al., 1995; Höfgen et al., 1995; Kim and Leustek, 2000). SAMS silencing could also cause translation to stall. SAM is the precursor of the polyamines spermine and spermidine, which play vital roles at many different stages of translation (Yoshida et al., 2001). A second possibility is that CGS or SAMS silencing causes the rate of amino acid synthesis to increase. Guyer et al. (1995) found that the expression of certain amino acid biosynthesis enzymes is increased in Arabidopsis when starved for a single amino acid. They proposed that Arabidopsis might contain a general amino acid control system analogous to the one regulated by the GCN4-transcription factor in yeast. Whether the loss of CGS or SAMS causes the rate of amino acid synthesis to increase has not yet been studied. A third possibility is that amino acids accumulate in response to growth inhibition or stress, which could negatively affect translation, amino acid transport, amino acid degradation, or the synthesis of amino acids necessary for secondary product formation (Zhao et al., 1998).

SAMS-silenced plants accumulate 250-fold more Met than wild type. This level is much higher than the 3- to 5-fold accumulation of other amino acids, indicating that Met-specific processes have been activated in the SAMS-silenced plants. There are several possible explanations for the increase in Met. The loss of SAMS would be expected to eliminate a major route for Met metabolism. Also, the rate of Met synthesis would be expected to increase for the following reasons: (a) TS is allosterically activated by SAM (Curien et al., 1998), thus TS activity is probably reduced in SAMS-silenced plants because of the likely reduction in SAM level; (b) CGS expression is increased by 3-fold (Fig. 7); and (c) the level of OPH is greater in SAMS-silenced plants (not shown). All of these effects would allow increased ability of CGS to direct OPH toward Met.

The pleiotropic effect of SAMS silencing on CGS expression is particularly intriguing because CGS level is increased despite the accumulation of extremely high Met levels. Previous results showed that application of Met to living plants causes a decline in CGS level (Thompson et al., 1982; Chiba et al., 1999). However, in these studies it was not clear whether CGS repression was caused by Met or one of its metabolites, e.g. SAM. The present result suggests that SAM, rather than Met, is probably the negative regulator of CGS expression. If so, it is tempting to speculate that SAM is the factor that mediates autogenous control of CGS expression mediated through the MTO1 region. An unfortunate deficiency of the present manuscript is that we have been unable to carry out reliable measurements of SAM. The inadequacy of presently existing methodologies for measurement of SAM from plant samples has been noted (Hanson and Roje, 2001). Nonetheless, to test the hypothesis that SAM is the effector of CGS expres-

sion in vivo, it will be essential to develop a sensitive and reliable method for use with Arabidopsis.

In this study, no attempt was made to discern the mechanism for CGS or SAMS silencing. However, both silencing events share the properties that they occur sporadically during development, are unstable, and are not meiotically stable. The meiotic instability is a hallmark of post-transcriptional gene silencing, a process that is influenced by the state of DNA methylation (Morel et al., 2000). In this regard it is interesting to note that changes in expression of both CGS and SAMS could potentially influence the state of DNA methylation.

MATERIALS AND METHODS

General Methods, Strains, Growth Media, and Growth Conditions

Molecular biology methods were performed as described by Sambrook et al. (1989). Protein concentration was measured using the Bradford method (Bio-Rad, Inc., Hercules, CA). *Agrobacterium tumefaciens* strain pGV2260 was used for transformation of Arabidopsis ecotype Colombia. *A. tumefaciens* was grown on yeast extract and peptone medium (10 g L^{-1} peptone, 10 g L^{-1} yeast extract, and 10 g L^{-1} NaCl). Arabidopsis plants were grown in vitro on agar medium, in potting mix, or in hydroponic medium. Growth on agar medium was under axenic conditions on 0.5× Murashige and Skoog nutrients (Life Technologies, Gaithersberg, MD) solidified with 8 g L^{-1} agar. Whenever supplements were added they are so indicated in the text. When grown on soil-less potting mix the seeds of segregating transgenic lines were transplanted after an initial selection on Kan-containing agar medium, or in the case of homozygous plants, were germinated directly on potting mix. The plants were fertilized at each watering with one-quarter-strength Peters (20:20:20, N:P:K) fertilizer (Grace-Sierra Co., Milpitas, CA) prepared in distilled water. Plants were grown hydroponically as described by Lee and Leustek (1998). All plants were incubated in growth chambers at 24°C with a diurnal cycle of 14-h light/10-h darkness. The light intensity was controlled at 100 μmol photons m^{-2} s^{-1}. Some experiments focused on transgenic Arabidopsis (Columbia) transformed with a construct for SAMS overexpression (Boerjan, 1993; Boerjan et al., 1994; de Carvalho et al., 1994).

Preparation of the CGS Transformation Construct and Transformation of Arabidopsis

A construct was prepared for stable overexpression of CGS in transgenic plants. The full-length *CGS* cDNA (Kim and Leustek, 1996; GenBank accession no. U43709) was cloned as an approximately 2-kb *Kpn*I-*Xba*I fragment into the same sites of pFF19 (Timmermans et al., 1990). Then an approximately 3-kb *Hin*dIII-*Eco*RI fragment including the cauliflower mosaic virus 35S promoter, *CGS* cDNA in the sense orientation, and the termination sequence was subcloned into the same sites of the binary vector pBI101. This construct was used to transform *A. tumefaciens*. Transformation of Arabidopsis was performed by vacuum infiltration (Bechtold and Pelletier, 1998). Transgenic T1 plants were selected by germination of seeds from the vacuum infiltrated plants on Murashige and Skoog medium with 50 μg mL^{-1} Kan.

Analysis of Transgenic Arabidopsis

The Kan-resistant plants were transferred to soil and their morphology observed throughout development. Analyses were conducted on plants taken up to the fourth transgenic generation. Plant tissues were analyzed by immunoblotting, CGS enzyme activity, PCR, and amino acid analysis. In addition to the analytical procedures, transgenic plants were tested for resistance to ethionine and PAG.

Ethionine and PAG resistance was studied by germinating seeds on Murashige and Skoog-agar medium supplemented with various concentrations of ethionine up to 50 μM or PAG up to 60 μM. Seedling growth was observed over time.

The transgene construct was detected in Kan-resistant plants by PCR (Lassner et al., 1989) using a 35S promoter-specific primer and a CGS-specific primer. The primer sequences were, 5′-TATCTCCACTGACGTAAGGGATGA-3′ and 5′-GGTCTAGAGAGAGAAGAGAACGAGAG-3′.

Immunoblotting was performed as described by Wang et al. (1993). The antibodies used in this study included one raised against Arabidopsis CGS (Kim and Leustek, 2000), an antibody against Arabidopsis SAT (Murillo et al., 1995), and an antibody raised against *Catharanthus roseus* SAMS (Schröder et al., 1997). Antibody complexes were detected on Kodak X-OMAT film (Rochester, NY) using the Renaissance Kit (PerkinElmer Life Sciences, Boston). The CGS antibody has previously been shown to accurately reflect the activity of CGS in transgenic plant tissues (Kim and Leustek, 2000). Unless stated otherwise, 5 μg of protein was analyzed. The CGS antiserum was used at 1:10,000 dilution, the SAT antiserum at 1:2,000 dilution, and the SAMS antiserum at 1:5,000 dilution.

CGS activity was measured in soluble protein extracts prepared from leaves in 20 mM MOPS-NaOH, pH 7.4. The assay was carried out with either OPH or *O*-succinylhomoserine either of which are used by CGS (Thompson et al., 1982; Ravanel et al., 1995). Unless noted otherwise OPH was used. The reaction mixture (100 μL) contained 20 mM MOPS-NaOH (pH 7.5), 1 mM dithiothreitol, 1 mM pyridoxal phosphate, 1 mM L-Cys, 0.44 mM OPH, and 50 μg of total protein. Reactions lacking OPH were incubated for 1 h, and then OPH was added and the incubation continued at 30°C; samples were taken for analysis at 0, 10, 20, and 30 min. The reaction was terminated by addition of 100 μL of 40 mM HCl. After centrifugation, 10 μL of the reaction was derivatized with AQC reagent (Waters, Inc., Milford, MA), and cystathionine was measured by HPLC as described below. When *O*-succinylhomoserine was used as a substrate the assay was performed as described by Ravanel et al.

(1995) and modified by Kim and Leustek (2000). All enzyme activities are the mean of three independent measurements ± SD.

For amino acid analysis, plants or plant tissues were weighed in microcentrifuge tubes, immediately frozen in liquid nitrogen, and stored at −80°C. All plant material was harvested 8 h into the light period. Frozen plant material was ground with a pestle to a fine powder in the microcentrifuge tube and was further homogenized at 5°C in 20 mM HCl using a ratio of 10 μL of HCl for each milligram of tissue. Norleucine was added as an internal standard. Tissue extracts were centrifuged for 10 min at 14,000*g*, 4°C. The supernatant was recovered and amino acids derivatized with 6-aminoquinolyl-*N*-hydroxysuccinimidyl carbamate (AQC; Cohen and van Wandelen, 1997) using an AccQ-Fluor Reagent Kit (Waters, Inc.). Ten microliters of the supernatant was added to 70 μL of borate buffer. The reaction was initiated by the addition of 20 μL of AQC reagent followed immediately by mixing and then incubation for 1 min at room temperature. Five microliters was injected onto the column. HPLC was performed using a Beckman model 126 solvent delivery system, autosampler, and 32 Karat System Gold data collection and analysis software (Beckman-Coulter, Fullerton, CA). Separations were performed on a 3.9- × 150-mm Waters Nova-Pak C_{18} column equipped with a Nova-Pak C_{18} Guard-Pak insert. Eluted amino acid-derivatives were detected using a Hitachi model F1080 Fluorescence Detector (Danbury, CT) with an excitation wavelength of 250 nm and an emission wavelength of 395 nm. Eluent A containing sodium acetate and triethylamine at pH 5.05 was purchased as a premix from Waters; eluent B was acetonitrile:water (60:40). The elution protocol was 0 to 0.5 min, 100% A; 0.5 to 5.0 min, linear gradient to 5% B; 5 to 35 min, linear gradient to 7.5% B; 35 to 41 min, linear gradient to 10% B; 41 to 44 min, 10% B; 44 to 54 min, linear gradient to 20% B; 54 to 61 min, 20% B; 61 to 71 min, linear gradient to 30% B; 71 to 86 min, linear gradient to 100% B. The flow rate was 1.0 mL/min. Standard solutions of each amino acid were analyzed at several concentrations to calculate a detector response factor. All amino acid standards were commercially obtained, except for OPH, which was synthesized and purified as described by Lee and Leustek (1999). SMM was also measured by the AQC method.

RNA was purified from 100 mg fresh weight of tissue by grinding in 1 mL of buffer containing 50 mM Tris-HCl (pH 8.0), 300 mM NaCl, 5 mM EDTA, 2 mM aurin tricarboxylic acid, 14 mM 2-mercaptoethanol, and 2% (w/v) SDS. The supernatant was recovered after centrifugation and RNA precipitated by addition of 0.4 mL of 8 M LiCl and incubation overnight at 4°C. After centrifugation, the pellet was dissolved in 0.4 mL of 2 mM aurin tricarboxylic acid and extracted with an equal volume of pH neutralized phenol. Finally, the RNA was precipitated with 50 mM sodium acetate and ethanol. RNA was electrophoresed on a 1% (w/v) agarose gel containing formaldehyde (Sambrook et al., 1989), blotted onto HyBond-N+ membrane (Amersham, Inc., Buckinghamshire, UK) and hybridized with a *CGS* cDNA probe. The highest stringency wash of the membrane was 0.1% (w/v) SSC, 0.1% (w/v) SDS at 65°C for 2 h. The blot was exposed to Kodak X-Omat film. The probe was prepared using a random primer labeling kit (Gibco-BRL, Inc., Cleveland) and [α-^{32}P]dCTP (111 TBq mmol^{-1}, New England Nuclear, Inc., Boston).

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Exhibit 5

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RESEARCH PAPER

High-level tryptophan accumulation in seeds of transgenic rice and its limited effects on agronomic traits and seed metabolite profile

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Abstract

Metabolic manipulation of plants to improve their nutritional quality is an important goal of plant biotechnology. Expression in rice (*Oryza sativa* L.) of a transgene (*OASA1D*) encoding a feedback-insensitive α subunit of rice anthranilate synthase results in the accumulation of tryptophan (Trp) in calli and leaves. It is shown here that the amount of free Trp in the seeds of such plants is increased by about two orders of magnitude compared with that in the seeds of wild-type plants. The total Trp content in the seeds of the transgenic plants was also increased. Two homozygous lines, HW1 and HW5, of *OASA1D* transgenic rice were generated for characterization of agronomic traits and aromatic metabolite profiling of seeds. The marked overproduction of Trp was stable in these lines under field conditions, although spikelet fertility and yield, as well as seed germination ability, were reduced compared with the wild type. These differences in agronomic traits were small, however, in HW5. In spite of the high Trp content in the seeds of the HW lines, metabolic profiling revealed no substantial changes in the amounts of other phenolic compounds. The amount of indole acetic acid was increased about 2-fold in the seeds of the transgenic lines. The establishment and characterization of these *OASA1D* transgenic lines have thus demonstrated the feasibility of increasing the Trp content in the seeds of rice (or of other crops) as a means of improving its nutritional value for human consumption or animal feed.

Key words: Amino acids, anthranilate synthase, AS, non-containment greenhouse, IAA, isolated field, 5MT, *OASA1D*, *Oryza sativa*.

Introduction

Metabolic manipulation of plants to improve their nutritional value is a primary goal of plant biotechnology. Essential amino acids such as Lys, Met, Thr, and Trp contribute substantially to the nutritional quality of plant-based foods for humans and domestic animals, but the amounts of such amino acids are limited in many crops. Dietary supplementation with Trp increases the growth rate of pigs and poultry (Subcommittee on Poultry Nutrition, 1994; Subcommittee on Swine Nutrition, 1998). An inadequate supply of Trp leads to a marked reduction in food intake in pigs as a result of the reduced synthesis of serotonin in the brain (Henry *et al.*, 1992; Sève, 1999; Eder *et al.*, 2001). Indeed, Trp has been used as a pharmaceutical agent in the treatment of depression (Massey *et al.*, 1998). The ability to increase the level of Trp in food crops by

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metabolic engineering is thus desirable from both nutritional and clinical viewpoints. The achievement of an increase in the amino acid content of seeds by genetic engineering, however, has been limited to Lys (Falco *et al.*, 1995; Mazur *et al.*, 1999; Zhu and Galili, 2003) and Met (Movig *et al.*, 1997; Lai and Messing, 2002).

The accumulation of free Trp in plants has been achieved by the introduction of genes encoding feedback-insensitive α subunits of anthranilate synthase (AS), which catalyses the conversion of chorismate to anthranilate. This approach has thus yielded increased levels of Trp in the roots of the forage legume *Astragalus sinicus* (Cho *et al.*, 2000) and in the leaves of tobacco (Zhang *et al.*, 2001). A mutant *OASA1* gene, *OASA1D* [formerly referred to as *OASA1(D323N)*], that encodes a feedback-insensitive α subunit of rice AS (Tozawa *et al.*, 2001) has previously been generated. Rice calli and leaves as well as potato plants and tubers that express *OASA1D* accumulate large amounts of free Trp (Tozawa *et al.*, 2001; Yamada *et al.*, 2004).

Manipulation of metabolic pathways in plants by genetic engineering thus has the potential to improve the nutritional value of crops and to allow the production of desired natural products in higher plants (DellaPenna, 2001; Morandini and Salamini, 2003). The proteins encoded by the introduced genes might affect not only the abundance of the target metabolite, however, but also, through changes in metabolic networks, that of related compounds. Moreover, such changes may influence the physiology or morphology of plants. Little is known, however, of the consequences of such manipulation for metabolite profiles and agronomic traits in plants.

Metabolism of Trp in plants is associated with the generation of a range of secondary compounds such as indole alkaloids and indole acetic acid (IAA). Manipulation of the Trp biosynthetic pathway might thus be expected to influence the synthesis of such metabolites and thereby elicit a pronounced change in metabolite profile. Indeed, overexpression of Trp decarboxylase, which catalyses the conversion of Trp to tryptamine, resulted in a marked reduction in the amount of indole glucosinolate in canola plants (Chavadej *et al.*, 1994) and a decreased abundance of Trp, Phe, and chlorogenic acid in potato (Yao *et al.*, 1995).

Evaluation of transgenic plants of improved nutritional value for effects of the transgene on agronomic traits and metabolite profiles is thus essential. However, the possible effects of the accumulation of Trp or of any other essential amino acid in seeds on agronomic traits and metabolite profiles have not been determined to date. Demonstration that Trp is the only major metabolite that accumulates in transgenic rice seeds, for example, would be likely to increase their acceptability for consumption by humans and farm animals.

The effects of *OASA1D* expression in rice both on the amounts of Trp and other amino acids in seeds and on agronomic traits of plants cultivated in isolated field trials have now been studied. The profile of phenolic compounds as well as the content of IAA in the transgenic seeds was also determined. Rice is one of the major crops in the world and its use for animal feed has recently been developed (Sakai *et al.*, 2003*a*). The results of the increase of Trp in transgenic rice prove the usefulness of the gene for improving the nutrition of other crops for animal feed.

Materials and methods

Plant materials and growth conditions

Seed calli of rice (*Oryza sativa* L. cv. Nipponbare) were transformed with the use of *Agrobacterium* as described by Hiei *et al.* (1994). The generation of rice plants transgenic for *OASA1D* or *OASA2*, with both genes controlled by the promoter of the maize ubiquitin gene, has also been described (Tozawa *et al.*, 2001; Yamada *et al.*, 2004). Rice plants transgenic for the β-glucuronidase gene (*GUS*) were also generated as described by Urushibara *et al.* (2001). All transgenic plants contained the hygromycin phosphotransferase gene (*hpt*) under the control of the 35S promoter of the cauliflower mosaic virus. Transgenic rice plants (R0 to R4) and non-transformed control plants (Nipponbare) were grown at 28 °C and 60% humidity under natural light conditions in pots (1/10 000a) containing podosol soil (Sumitomo Chemical, Osaka, Japan) in a containment greenhouse.

Two *OASA1D* transgenic lines, HW1 and HW5, were also grown in a non-containment greenhouse (R5) and an isolated field (R6). In 2002, seeds of the HW lines and Nipponbare were sown on 15 April and the seedlings were transferred individually to pots (1/10 000a) containing podosol soil on 15 May. The plants were maintained in the non-containment greenhouse under natural light and temperature conditions at the National Institue of Crop Science (NICS). No additional fertilizer was applied. In 2003, seeds of the HW lines and Nipponbare were sown on 14 May and the seedlings were transferred to an isolated paddy field (10 m×1.2 m) at the National Institute for Agro-Environmental Sciences in Tsukuba on 4 June. For each line, 40 seedlings were planted in duplicate in an area of 15 cm×30 cm per plant. The total amount of fertilizer applied m^{-2} included 6 g of N, 6 g of P_2O_5, and 6 g of K_2O and was added at planting. Other practices followed the cultivation standards of Ibaraki Prefecture (Ibaraki Prefecture Standard Lowland Rice Cultivation, 1990).

Evaluation of agronomic traits

Agronomic traits, including heading date, culm length, and morphological characteristics, were evaluated by standard protocols (Ibaraki Prefecture Standard Lowland Rice Cultivation, 1990). Plants in the isolated field were harvested individually, air-dried, and analysed for yield and seed germination. All panicles were harvested from each plant, dried at 38 °C for 4 weeks, and then maintained in a freezer at −30 °C until analysis of amino acids and of IAA and metabolite profiling. For germination analysis, 15 or 20 seeds of individual lines were transferred to a filter paper that had been moistened with distilled water and placed in a Petri dish (6 cm in diameter); the analysis was performed in duplicate. The Petri dish was maintained at 35 °C for 15 d in the dark, and seed germination was assessed each day according to the modified standard method of NICS based on viviparity (Sakai *et al.*, 2003*b*).

Amino acid analysis and determination of total nitrogen content

Dehulled seeds were autoclaved individually with 50 µl of water in a 1.5 ml Eppendorf tube for 15 min, and extracts were prepared from each seed as previously described (Wakasa and Widholm, 1987). The

amounts of free amino acids were then quantified with the use of the PICO-TAG analysis system (Waters, Milford, MA). For quantitation of total Trp, five dehulled grains were pulverized with a mortar and pestle and heated in 5 M NaOH for 28 h at 110 °C; the hydrolysate was then acidified with 6 M HCl and subjected to analysis with an L-8800 High Speed Amino Acid Analyser (Hitachi High-Technologies, Tokyo, Japan).

Dehulled rice seeds were weighed and decomposed in concentrated sulphuric acid in the presence of salicylic acid and sodium thiosulphate. The ammonia formed was distilled, and was determined colorimetrically at 640 nm using the indophenol method.

Metabolite profiling

Four dehulled seeds were pulverized with a mortar and pestle and subjected to extraction for 1 h with 10 vols (v/w) of a mixture of water:methanol:acetic acid (249:250:1, by vol.). The extract was centrifuged at 16 000 *g* for 20 min, the resulting supernatant was passed through a SepPak C_{18} cartridge (Waters, Milford, MA), and the eluate was subjected to reversed-phase HPLC (LC-10Avp system; Shimadzu, Kyoto, Japan) with a Cadenza Column CD-C18 [250 mm×4.6 mm (inner diameter); Imtakt, Kyoto, Japan). Elution was performed with a mixture of acetonitrile and 0.02% aqueous trifluoroacetic acid (3:97 v/v, at 0 min; 30:70 v/v at 40 min; 98:2 v/v at 75 min) at a flow rate of 0.85 ml min^{-1} and a temperature of 40 °C; it was monitored with a photodiode array detector (Shimadzu SPD-M10Avp) over a wavelength range of 190–400 nm.

IAA analysis

Six dehulled seeds were pulverized with a mortar and pestle and then soaked for 3 h at 4 °C in 10 vol (v/w) of 80% acetone in water containing 2.5 mM diethyl dithiocarbamate. This extraction procedure was repeated three times. The combined extract was divided into three portions that were respectively subjected to quantification of free IAA, free plus ester forms of IAA and total IAA. The free IAA in the extract was partially purified by solid-phase extraction and quantified by liquid chomatography and tandem MS (LC-MS/MS) as previously described by Matsuda *et al.* (2005). For determination of the amount of free plus ester forms of IAA, the original extract was acidified to pH 2 with aqueous HCl and then analysed as for free IAA. For determination of the amount of total IAA, the extract was subjected to hydrolysis with 7 M NaOH for 3 h at 100 °C under N_2 before analysis. [Phenyl-$^{13}C_6$]IAA was used as the internal standard in these analyses.

Results and discussion

Generation of rice plants expressing OASA1D

Transgenic rice plants that express *OASA1D* were generated and subjected to the analyses of Southern blot and northern blot as described previously (Tozawa *et al.*, 2001; Yamada *et al.*, 2004). Given that expression of *OASA1D* confers resistance to 0.3 mM 5-methyltryptophan (5MT), some transgenic plants were generated from calli selected with 5MT instead of with hygromycin (Yamada *et al.*, 2004). Transgenic lines selected by growth in the presence of 5MT or hygromycin are denoted by M or H, respectively.

Spikelet fertility of greenhouse-grown plants

Almost all regenerated plants of >120 transgenic lines grown in pots exhibited normal growth, with exceptional instances of dwarfism or slow growth presumably being attributable to somaclonal mutations (Phillips *et al.*, 1994). However, for plants grown in a greenhouse, the spikelet fertility of transgenic plants tended to be lower than that of the wild type (Table 1). The mean spikelet fertility of the transgenic plants was thus only 31%, compared with a value of 76% for seed-grown Nipponbare. Given that a decrease in spikelet fertility has previously been observed in transgenic rice plants generated by tissue culture (Hiei *et al.*, 1994; Urushibara *et al.*, 2001), part of the reduction in spikelet fertility apparent in *OASA1D*-expressing plants might be due to the regeneration process. However, transgenic rice plants that overexpress the wild-type gene (*OASA2*) for another α subunit of rice AS but do not accumulate Trp in calli or leaves (Tozawa *et al.*, 2001) exhibited a spikelet fertility (61%) higher than that of plants that express *OASA1D*, but lower than that of Nipponbare, suggesting that accumulation of Trp might also contribute to the reduced spikelet fertility of the *OASA1D* transgenic plants. Analysis by genomic Southern hybridization of R0 (regenerated) plants of *OASA1D* transgenic lines with a spikelet fertility of >50% revealed that they had a relatively high copy number (three to seven) of the transgene (data not shown), suggesting that the copy number was not a principal cause of low spikelet fertility.

Trp content of seeds produced by greenhouse-grown plants

Seeds of 12 *OASA1D* transgenic lines with a spikelet fertility of >50% were analysed for free and total Trp contents in the second or third generation (R2 or R3 seeds) of plants grown in a greenhouse. All lines showed a marked increase in the amount of free Trp, with the mean free Trp content ranging from 3037 to 23 705 nmol g^{-1} of dry seed weight (Table 2); these values correspond to increases of 55- to 431-fold compared with the free Trp content of

Table 1. *Spikelet fertility of* OASA1D *transgenic plants (R0) grown in a greenhouse*

Transgene	Trp content	Spikelet fertility (%)						Total no. of plants
		≤20	21–40	41–60	61–80	81–100	Mean ±SD	
OASA1D	Increased	25	19	12	7	0	30.7±21.2	63
OASA2	Normal	5	4	6	14	11	61.4±27.2	40
NB[a]	Normal	0	1	2	3	6	76.2±19.7	12

[a] Seed grown Nipponbare plants.

Table 2. *Total and free Trp contents of seeds from* OASA1D *transgenic plants grown in a greenhouse*

Five seeds of one panicle from each transgenic line were used for measurement of total Trp content and of average seed weight. Total Trp content and seed weight of Nipponbare were determined as average values for two plants. Free Trp was measured in three to five single seeds, and values presented are means ±SD. Trp content is shown as nmol Trp g^{-1} dry seed weight.

Transgenic line	Seed generation	Seed weight (mg)	Total Trp (nmol g^{-1})	Free Trp (nmol g^{-1}) Means ±SD
Nipponbare		18.6	4188	55.0±21.7
GUS16[a]	R2	14.2	5173	41.0±8.0
M25	R2	11.7	8100	
M121	R3	14.9	8810	3036.6±233.4
M31	R2	17.1	9237	
M21	R2	9.9	9264	
H29	R2	14.1	11 320	
H36	R2	17.0	14 425	7642.3±830.3
H17	R3	16.2	14 178	12 309.0±5764.9
H37	R2	14.0	15 619	
H42	R2	11.9	16 967	
H40	R2	12.7	17 322	
M45	R2	12.7	20 830	14 495.3±3528.0
M34	R2	7.7	48 519	23 705.3±11 681.2

[a] Transgenic line harbouring *GUS* and *hph* (*OASA1D* lines contain *OASA1D* and *hph*).

non-transgenic Nipponbare seeds (55 nmol g^{-1}). The amount of free Trp as a percentage of total Trp in the transgenic seeds (34–87%) was also greatly increased compared with that in wild-type seeds (1.3%). Given that seeds of a transgenic line expressing *GUS*, which encodes β-glucuronidase, exhibited a Trp content similar to that of Nipponbare seeds, it was unlikely that the Trp accumulation apparent in seeds of *OASA1D* transgenic plants resulted from an abnormality caused by the transformation process.

The transgenic seeds analysed for Trp content were a mixture of those homozygous or heterozygous for *OASA1D*. To examine the possible influence of genotype on Trp content, seeds from a single transgenic plant of the H17 line in the R1 generation were divided into two groups. One group of 26 seeds showed segregation of *OASA1D* by PCR analysis (14 positive, 12 negative). The other group of 32 seeds, which also should have been a mixture of transgene genotypes, all contained an increased level of free Trp (9134±3740 nmol g^{-1}). These results thus indicated that Trp accumulation in seeds is determined primarily by the genotype of the mother plant. However, given that the free Trp content of seeds of the H17 or M34 lines showed some variability (Table 2), the level of free Trp in seeds might also be influenced by seed genotype. Although the physiological conditions of plants and seeds also affect amino acid content, the increase in the amount of Trp in the seeds of transgenic rice expressing *OASA1D* was sufficiently high to be attributed to the activity of the transgene.

It is the total Trp content, including both free Trp and Trp in proteins, that is important for the nutritional value of seeds. The increase in the amount of free Trp in seeds of *OASA1D* transgenic plants was accompanied by an increase in the total Trp content (Table 2). The total Trp content of seeds of the various transgenic lines thus ranged from 8100 to 48 519 nmol g^{-1}, values that correspond to increases of 1.9- to 11.6-fold compared with that for Nipponbare (4188 nmol g^{-1}).

The weight of individual dehulled seeds tended to be smaller for *OASA1D* transgenic lines than for Nipponbare (Table 2). However, a reduced seed weight was also apparent for plants harbouring a *GUS* transgene, suggesting that this effect might be attributable to the transformation process. *OASA1D* transgenic line M34, whose seeds showed the highest Trp content, also manifested the lowest seed weight. However, the seeds of line M21, which exhibited a medium level of Trp accumulation, were also of low weight. No clear correlation between Trp content and seed weight was thus apparent.

Generation of homozygous OASA1D *transgenic rice lines for evaluation of agronomic traits*

Two *OASA1D* transgenic lines, M121 and H41, were advanced to obtain homozygotes for evaluation of agronomic traits. The resulting homozygous lines were designated HW1 for M121 and HW5 for H41. The early generations of these homozygous lines exhibited a spikelet fertility of >50% and normal morphological features. Genomic Southern blot analysis of selfed progenies and of F_2 plants of F_1 hybrids between either HW1 or HW5 and Nipponbare revealed that HW1 contained three copies of the transgene and HW5 harboured four copies. No segregation of hybridized bands was observed in F_2 plants, indicating that the transgenes were integrated at one locus (data not shown).

Trp content of seeds produced by field-grown OASA1D *transgenic plants*

The free and total Trp contents as well as the nitrogen content of R5 and R6 seeds of the HW lines grown in a greenhouse were higher than those of Nipponbare seeds (Table 3), and all of these values were slightly higher for HW1 than for HW5. The levels of free and total Trp in seeds were stable during growth of the HW lines in a greenhouse for 2 years (data not shown). In the field condition, the free Trp content of seeds of the HW lines was increased about 2-fold compared with the corresponding values for seeds of greenhouse-grown plants (Table 3). This increased accumulation of free Trp in the seeds of field-grown plants was not accompanied by a similar increase in the total Trp content. The level of total Trp in seeds of the HW lines was thus stable under different growth conditions. The nitrogen content of seeds of field-grown HW lines and Nipponbare was lower than the corresponding values for seeds of greenhouse-grown plants. The amounts

of free and total Trp in Nipponbare seeds were also lower in the field condition than in the greenhouse, with the result that the relative values to Nipponbare for seeds of the field-grown HW lines were increased accordingly (Table 3). The levels of Trp in the seeds of Nipponbare grown in another field were similarly low (data not shown).

The opposite effects of field growth on the free Trp content of seeds of the HW lines and of Nipponbare seeds might be attributable, in part, to a response of the transgene promoter to the cooler temperatures or to the difference in temperature between night and day in the field. The *OASA1D* gene was driven by the ubiquitin gene promoter, which is responsive to stress (Takimoto *et al.*, 1994) and might therefore be activated by low temperatures, resulting in increased expression of *OASA1D* and a greater accumulation of free Trp.

Amino acid composition of seeds produced by field-grown OASA1D *transgenic plants*

The increased Trp content of rice calli expressing *OASA1D* does not result in substantial changes in the amounts of other amino acids (Tozawa *et al.*, 2001). The marked accumulation of free Trp in the seeds of the HW1 and HW5 lines grown under field conditions was accompanied by an increase in the amounts of other amino acids to some extent (Table 4). Their increases were relatively small compared

Table 3. *Nitrogen and Trp contents of seeds produced by HW lines under field or greenhouse conditions*

Five seeds of Nipponbare (NB) or HW lines were analysed for nitrogen content (percentage of dry seed weight) and total Trp content (nmol g^{-1} dry seed weight) in each of the indicated (in parentheses) number of experiments, and data presented are means ±SD. Values for free Trp content (nmol g^{-1} dry seed weight) are presented as means ±SD for the numbers of seeds indicated in parentheses. Means followed by the same letters are not significantly different at the 5% level (Student's *t* test).

Growth condition	Line	N content (%)	Free Trp		Total Trp	
			(nmol g^{-1})	R.V.[a]	(nmol g^{-1})	R.V.[a]
Greenhouse	HW 1	1.39±0.12 (2)	2403±283.4 a (6)	55.9	7883±151.2 a (3)	1.9
	HW 5	1.30±0.10 (2)	1762±333.3 b (6)	41.0	6949±387.6 b (3)	1.7
	NB	1.24±0.04 (2)	43±15.7 c (6)	1	4118±237.0 c (3)	1
Field	HW1	1.13±0.03 (2)	5294±2672.6 a (6)	311.0	8477±1139.0 a (4)	14.1
	HW5	1.04±0.01 (2)	3288±1366.1 a (6)	193.0	5684±1818.0 b (4)	9.4
	NB	0.96±0.13 (2)	17±9.4 b (9)	1	602±413.0 c (3)	1

[a] Relative value compared with Nipponbare.

Table 4. *Free amino acid composition of seeds produced by field-grown HW lines*

Data are expressed as nmol of amino acid g^{-1} dry seed weight and are means ±SD of values from six seeds of Nipponbare (NB) or HW lines. Means followed by the same letters are not significantly different at the 5% level (Student's *t* test).

Amino acid	Free amino acid content (nmol g^{-1})							
	NB		HW1			HW5		
	Mean ±SD	(%)[a]	Mean ±SD	(%)[a]	Relative value[b]	Mean ±SD	(%)[a]	Relative value[b]
Gly	51±29 a	1.9	153±95 b	1.4	3.0	142±26 b	1.8	2.8
Ala	154±95 a	5.8	794±581 b	7.3	5.2	608±315 b	7.9	3.9
Val	32±19 a	1.2	84±37 b	0.8	2.6	68±16 b	0.9	2.1
Leu	13±7 a	0.5	36±20 b	0.3	2.7	32±8 b	0.4	2.5
Ile	8±5 a	0.3	28±15 b	0.3	3.5	26±9 b	0.3	3.3
Ser	172±95 a	6.5	288±107 a	2.6	1.7	277±68 a	3.6	1.6
Thr	37±30 a	1.4	70±37 a	0.6	1.9	57±31 a	0.7	1.5
Met	5±4 a	0.2	8±2 a	0.1	1.6	6±2 a	0.1	1.2
Asp	575±333 a	21.9	1285±727 a, b	11.7	2.2	1136±139 b	14.7	2.0
Asn	595±760 a	22.6	1269±616 a	11.6	2.1	746±296 a	9.7	1.3
Glu	660±359 a	25.1	810±303 a	7.4	1.2	739±165 a	9.6	1.1
Gln	51±31 a	1.9	47±13 a	0.4	0.9	54±8 a	0.7	1.1
Arg	67±71 a	2.5	239±123 b	2.2	3.6	102±48 a	1.3	1.5
Lys	16±7 a	0.6	65±34 b	0.6	4.1	48±14 b	0.6	3.0
His	28±17 a	1.1	115±48 b	1.1	4.1	116±75 b	1.5	4.1
Phe	7±4 a	0.3	30±15 b	0.3	4.3	25±7 b	0.3	3.6
Tyr	91±55 a	3.5	208±74 b	1.9	2.3	171±28 b	2.2	1.9
Trp	20±9 a	0.8	5294±2673 b	48.4	264.7	3288±1366 b	42.5	164.4
Pro	49±31 a	1.9	125±60 b	1.2	2.6	96±17 b	1.2	2.0
Total	2629±2001 a	100	10 947±4484 b	100	4.2	7734±1810 b	100	2.9

[a] Amount of each amino acid as a percentage of total amino acids.
[b] Amount of each amino acid relative to that in Nipponbare.

with that of Trp. The total amount of free amino acids was increased 4.2- and 2.9-fold in HW1 and HW5, respectively, compared with the value for Nipponbare, with the maximal change in the content of any one amino acid (other than Trp) being limited to a 5.2-fold increase. However, with the exception of Ala (and Trp), the ratio of the amount of each amino acid to the total amount of free amino acids was reduced or remained virtually the same in the transgenic lines compared with Nipponbare.

The levels of Lys and Phe were low in Nipponbare seeds but their absolute amounts were increased in the HW seeds, with the result that their percentage contributions to the total amount of free amino acids were the same in the HW lines and in the wild type. Given that Gln is the amino donor for the synthesis of anthranilate, its abundance might have been expected to be changed in the seeds of the transgenic lines. Its absolute amount in seeds of the HW lines was similar to that in Nipponbare seeds, however. Serine is also a precursor for Trp synthesis, but its amount in HW seeds was not significantly increased compared with that in Nipponbare seeds.

The increases in the absolute amounts of free amino acids in the seeds of the transgenic lines suggest the existence of regulatory mechanisms that increase amino acid synthesis in response to Trp accumulation. The *opaque-2* mutation in maize (Oh545*o2*) is associated with an increased level of free amino acids in mature endosperm (Wang and Larkins, 2001). Genetic analysis suggests that the gene for aspartate kinase 2 is the gene responsible for the effect of this mutation on free amino acid content (Wang *et al.*, 2001). A mutation in a transcriptional regulator of AS genes also renders Trp biosynthesis insensitive to Trp concentration (Bender and Fink, 1998). Our results therefore suggest that Trp accumulation in rice seeds might increase the transcription of genes that encode enzymes responsible for amino acid synthesis.

Agronomic traits of field-grown OASA1D *transgenic plants*

HW lines grown in a greenhouse appeared similar to Nipponbare with regard to most agronomic traits analysed (Table 5). For plants grown under field conditions, however, differences in traits related to seed productivity were apparent between the transgenic lines (especially HW1) and the wild type. Both HW lines grown in the field thus exhibited a spikelet fertility lower than that of Nipponbare, although pollen fertility (Table 5) and anther size (reflecting the number of pollen grains) (data not shown) for the transgenic lines were similar to those for Nipponbare. Moreover, the average spikelet number per panicle was significantly smaller for the HW lines than for Nipponbare. The reduction in the number of spikelets per panicle and the low spikelet fertility likely contributed to an observed decrease in harvested seed weight for HW1 and HW5 to 52.5% and 69.6% of the value for Nipponbare, respectively. The harvested plant weight was similar for the HW lines and Nipponbare (data not shown). Whereas the individual brown seed weight varied among transgenic lines in early generations (Table 2), it did not differ markedly among HW lines and Nipponbare under field conditions (Table 5).

The high concentration of Trp in the HW lines is likely to be the primary cause of the differences in agronomic traits between these lines and Nipponbare grown under field conditions. Spikelet number per panicle is determined at an early stage of development of the inflorescence apex and is influenced by several conditions, such as nitrogen and carbon availability as well as temperature (Takeoka *et al.*, 1993). Accumulation of Trp might increase the sensitivity of plants of the HW lines to environmental stress and thereby reduce spikelet number and fertility in the field condition.

The culm length of HW lines grown in the field was smaller than that of Nipponbare, although this difference

Table 5. *Agronomic traits of HW1 and HW5 lines*

Means followed by the same letters are not significantly different at the 5% level (Student's *t* test).

Growth condition (year)	Line	n^a	Days to heading ±SD	Culm length (cm)	Panicle length (cm)	No. of tillers plant^{-1}	Spikelet fertility (%) ±SD[b]	No. of spikelets panicle^{-1}	Mean dry matter of harvested seeds (30 plants g^{-1}) ±SD[c]	Pollen fertility (%)[d]	Brown seed weight (1000 grains g^{-1})[e]
Greenhouse (2002)	HW1	10	129±0.64	78.1 a	16.1 a	13.7 a	79.5±6.84			98.3	20.1 a
	HW5	10	132±2.04	74.9 a	15.7 a	13.1 a	88.3±5.19			97.5	20.1 a
	NB	23	130±1.13	80.1 a	17.0 a	11.6 a	88.2±6.65			98.9	20.1 a
Field (2003)	HW1	2	100±0.00	73.3 b	18.1 a	15.5 a	65.0±18.21	71.6 b	443.6±35.64	97.6	20.2 ab
	HW5	2	101±0.50	75.7 ab	17.8 a	13.2 a	81.3±11.00	85.6 b	588.4±50.77	98.5	21.0 a
	NB	2	100±0.00	81.3 a	20.1 a	12.2 a	91.6±2.91	122.3 a	844.8±27.86	99.4	20.0 b

[a] *n* means repetition. The number of plants or plots for the greenhouse and field conditions, respectively. Heading date, culm length, panicle length, and the number of tillers were recorded for each of 10 or 23 plants in the greenhouse condition and for each of 15 plants per plot in the field condition.
[b] Spikelet fertility was recorded for all panicles of 10 plants in 2002 and for three panicles of main culms of 60 plants in 2003.
[c] Mean dry matter of harvested seeds which were air-dried (assumed moisture content of seeds was approximately 10–15%).
[d] Pollen fertility was recorded for three spikelets of each of three randomly selected plants. It was estimated from microscopic analysis of the shape and color of the pollen stained with I-KI solution (1%).
[e] The weight of 1000 seeds was determined from that of 20 or 100 grains in the greenhouse and field conditions, respectively.

was statistically significant only for HW1 (Table 5). No difference in plant height was observed between HW lines and Nipponbare (data not shown). A short culm length has often been observed in plants regenerated from tissue culture (Phillips *et al.*, 1994), suggesting that this characteristic of the HW lines might be attributable to somaclonal mutation.

Germination of seeds from field-grown OASA1D *transgenic plants*

The growth condition markedly influenced seed germination in HW lines. Whereas the germination percentage for seeds of greenhouse-grown plants was similar for HW lines and Nipponbare, it was greatly reduced for seeds of field-grown HW1 plants compared with that for field-grown HW5 or Nipponbare plants (Table 6). The time to germination was also increased for seeds from both HW lines grown under field conditions, as well as for seeds from HW1 plants grown in the greenhouse.

Poor seed germination has been observed in other plants with increased levels of an essential amino acid. Transgenic soybean with a large increase in free Lys content thus manifested reduced seed viability (Falco *et al.*, 1995). Seeds of transgenic *Arabidopsis thaliana* with a high Lys content also exhibited retarded germination and seed establishment (Zhu and Galili, 2003). The seeds of HW1 plants grown in the field showed the lowest frequency of and greatest delay in germination as well as the highest content of free Trp compared with seeds of HW1 or HW5 plants grown in the greenhouse and HW5 plants grown in the field. These observations suggest that an increase in Trp content over a certain threshold level might substantially influence germination. The importance of amino acid metabolism such as Gln, Lys, and Met in maize germination efficiency has been shown (Limami *et al.*, 2002; Anzala *et al.*, 2006). Trp has been shown to be an endogenous inhibitor of embryo germination in white wheat (Morris *et al.*, 1988).

It might prove possible to ameliorate the unfavourable traits observed in the HW lines grown under field conditions by controlling the extent and tissue distribution of Trp accumulation with the use of a different promoter to drive *OASA1D* expression. Potential promoters for this purpose include those of embryo-specific genes or of genes that are not responsive to stress.

Table 6. *Germination percentage and time to seedling establishment for HW lines*

The germination test was performed with 20 seeds from greenhouse-grown plants and 15 seeds from field-grown plants. Data are means of values from two experiments.

Line	Greenhouse		Field	
	Germination (%)	Time to establish seedlings (d)	Germination (%)	Time to establish seedlings (d)
HW1	95	6.3	53.3	10.1
HW5	100	3.1	93.3	5.3
NB	100	3	100	2.8

Metabolite profile of seeds from field-grown OASA1D *transgenic plants*

Changes in the composition of aromatic components in the seeds of field-grown HW1 and HW5 plants were analysed by reversed-phase HPLC. Elution was monitored with a photodiode array detector over a wavelength range of 190–400 nm. Typical chromatograms obtained at 280 nm, the most effective wavelength for detection of changes in the composition of anthranilate-related metabolites, are shown in Fig. 1. The aromatic metabolite profiles of dehulled seeds revealed no apparent marked accumulation of components other than Trp in the transgenic seeds (Fig. 1, inset). Magnification of the chromatograms revealed small differences between HW lines and Nipponbare (Fig. 1). A peak with a retention time of 10 min, for example, was specifically detected in both transgenic lines. Essentially, identical results were obtained by monitoring elution at wavelengths other than 280 nm (data not shown). These results are surprising given the high levels of Trp in the transgenic seeds and that the Trp biosynthetic pathway gives rise to various secondary metabolites, such as the indole alkaloids and indole glucosinolates, in many plants. To date, no remarkable secondary metabolites of Trp origin

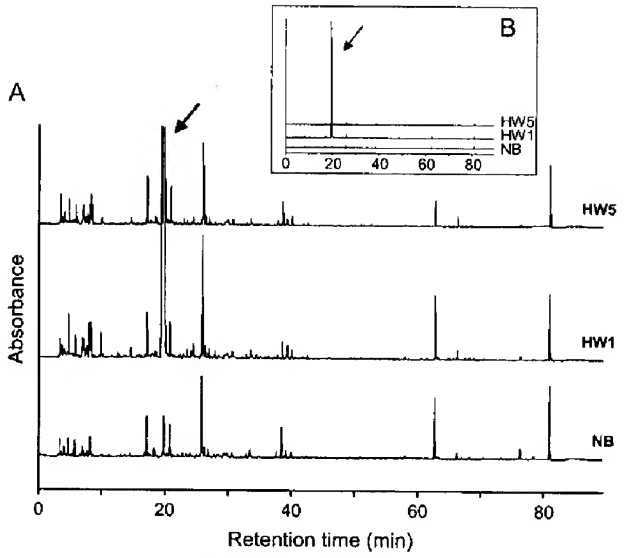

Fig. 1. Aromatic metabolite profiles of seeds derived from field-grown HW lines. Seed extracts of HW1, HW5, and Nipponbare were analysed by reversed-phase HPLC with detection at 280 nm. The traces in the inset are shown at higher magnification in the main panel. The arrow indicates the peak corresponding to Trp.

have been reported in rice plants and the present study suggested that the absence of such Trp-derived secondary metabolites was probably not due to a shortage of Trp supply, but to a very low capability of relevant Trp utilization in rice. Furthermore, Trp decarboxylase probably plays more important role in the divergence of Trp-related carbon flow into the secondary metabolism, as has been observed in transgenic plants overexpressing Trp decarboxylase (Chavadej *et al.*, 1994; Yao *et al.*, 1995)

It was specifically investigated whether the accumulation of Trp in the seeds of the field-grown HW lines affected the amount of IAA, given the close relations between the Trp biosynthetic pathway and IAA production. Therefore the levels of free IAA, of free IAA plus its ester conjugates, and of total IAA (including amide conjugates) were measured in the seeds of the HW lines and Nipponbare (Fig. 2A). The amounts of free and conjugated forms of IAA were each increased about 2-fold in the seeds of both HW lines compared with those in Nipponbare seeds. The increase in the level of free IAA in seeds was consistent with our previous demonstration of IAA accumulation in rice calli expressing *OASA1D* (Morino *et al.*, 2005). Increased auxin content has been associated with Trp accumulation in cultured carrot and potato cells resistant to 5MT (Widholm, 1977; Sung, 1979). The level of IAA conjugates was also found to be increased in the *Arabidopsis* mutant *Amt1*, which expresses a feedback-insensitive AS and accumulates Trp (Kreps and Town, 1992; Ludwig-Muller *et al.*, 1993). In addition, 5MT-resistant mutants of *Lemna gibba* showed an approximately 3-fold increase in the amount of free IAA (Tam *et al.*, 1995). Seeds of rice and maize normally contain higher concentrations of IAA than do other organs of these plants (Bandurski and Schulze, 1977). The increase in the amounts of free and total IAA apparent in the seeds of the HW lines thus suggests that rice seeds are able to accumulate IAA to especially high levels.

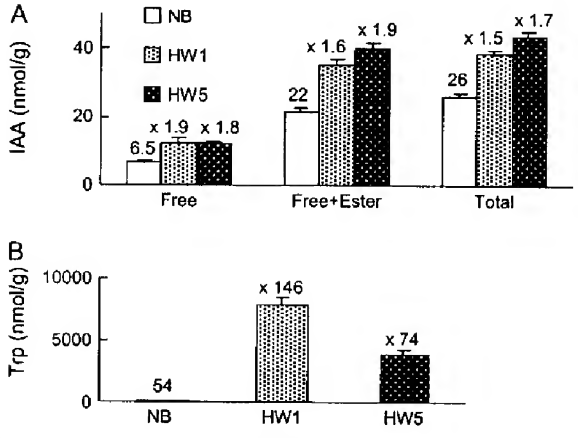

Fig. 2. IAA and Trp contents of seeds derived from field-grown HW lines. The amounts of IAA (A) and free Trp (B) were quantitated in the same seeds of HW1, HW5, or Nipponbare (NB) plants. Data are expressed as nanomoles of analyte per gram of dry seed weight and are means ±SD of values determined from three groups of six seeds.

Given that IAA affects multiple aspects of plant growth, the low spikelet fertility and density as well as the impaired seed germination of the HW lines might reflect the increased abundance of IAA rather than that of Trp. IAA markedly inhibited the germination of wheat embryos excised from caryopses that were highly dormant (Ramaih *et al.*, 2003); Trp, the precursor of IAA, was shown to be equally inhibitory in this instance, however. In the case of these HW lines, the impairment in seed agronomic traits was greater for HW1 than for HW5, whereas the amount of total IAA was slightly higher in the seeds of HW5 than in those of HW1. The impairment thus appeared to be more correlated with Trp content than with total IAA (Fig. 2). Regardless, the growth of HW1 and HW5 seedlings after germination overtook that of Nipponbare and no differences in plant growth at the harvesting stage were detected between Nipponbare and the HW lines (data not shown).

Conclusion

These results have revealed that the seeds of rice plants expressing the *OASA1D* transgene accumulate free Trp to high levels and in a stable manner. This accumulation of Trp was not accompanied by substantial changes in the amounts of other phenolic compounds. The growth of *OASA1D* plants under the field conditions used here revealed a reduction in spikelet fertility and in the number of spikelets per panicle, as well as in the efficiency of seed germination compared with Nipponbare. However, these differences with Nipponbare were more prominent in the HW1 line than in the HW5 line, the latter being largely similar to the wild type with regard to many agronomic traits.

For use as human food, it is preferable that transgenic crops do not contain antibiotic resistance genes. An advantage of *OASA1D* transgenic rice is that the transgene confers sufficient resistance to 5MT to allow the selection of transformed cells with this agent, thereby obviating the need for another gene as a selectable marker (Yamada *et al.*, 2004). Although rice is primarily grown for human consumption, its use for animal feed has been recently developed (Sakai *et al.*, 2003*a*). An increased Trp content of rice seed would thus also prove beneficial for animal nutrition.

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Exhibit 6

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Anaerobic Induction of Alanine Aminotransferase in Barley Root Tissue[1]

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ABSTRACT

Alanine aminotransferase, otherwise called glutamate-pyruvate aminotransferase (GPT), activity increases up to fourfold during several days of anaerobic induction in barley (*Hordeum vulgare* L.) roots, reaching a maximum activity of 13 international units per gram fresh weight. This increase in activity paralleled the increase in alcohol dehydrogenase activity in the same root tissue. Upon return to aerobic conditions, the induced GPT activity declined with an apparent half-life of 2 days. The isozyme profile of GPT in barley root tissue comprised one band of activity; in maize there were three bands of activity, the bands with greater mobility had much lower activity. Native polyacrylamide gel electrophoresis indicated that the induction of GPT activity results from an increase in the level of activity of these bands; no other activities were detected. When root tissue was induced under different levels of hypoxia (0%, 2%, 5%, and 21% O_2), changes in GPT activity were found to increase with lower levels of oxygen. Comparisons of GPT induction in barley, maize (*Zea mays*), rye, (*Secale cereale*) and wheat (*Triticum aestivum*) indicate that this enzyme is induced in the root tissue of all of these cereals; however, anaerobic root conditions do not result in the induction of GPT activity in leaf tissue. The dependence of GPT induction on high levels of nitrate in the media was tested by comparing activity levels in Hoagland solution and a nitrate-free nutrient solution. GPT activity was induced to similar levels under both conditions. These results indicate that alanine aminotransferase shows a very similar pattern of induction to alcohol dehydrogenase in barley root tissue and may be important in anaerobic glycolysis.

The flooding of soils can subject plant roots to considerable periods of anaerobiosis (5). This condition precludes aerobic respiration so that root survival becomes dependant on fermentative metabolism. Under these conditions, the synthesis of most cellular proteins is suppressed and a subset of anaerobic proteins are synthesized (18). These anaerobic proteins include ADH,[3] pyruvate decarboxylase (14), LDH (11), and several other glycolytic enzymes (12, 13). During periods of O_2 deficiency, plants produce a number of glycolytic end products including ethanol, lactate, various organic acids, and amino acids (2, 3).

Although large amounts of ethanol are produced by anaerobic roots, one of the major products of anaerobic metabolism in root tissue is alanine. Effer and Ranson (4) showed that the levels of alanine increased fourfold after 12 h of anaerobic treatment in buckwheat seedlings. Smith and apRees (21) and Hoffman *et al.* (11) have shown by radiolabeling studies that pea and barley roots produce large amounts of alanine under anaerobic conditions. The *in vivo* NMR data of Roberts *et al.* (17) also demonstrate that, even during the initial stages of anaerobiosis (0–120 min), alanine begins to accumulate in root tissue.

Pyruvate is converted to alanine in plant cells by alanine aminotransferase (EC 2.6.1.2) (GPT, 7). The occurrence of this enzyme has been reported in a number of different plants and plant tissues (1, 9, 15, 22). Hatch and Mau (10) found that a number of C_4 pathway species contain exceptionally high activities of aspartate and alanine aminotransferases. Two major isozymes of alanine aminotransferase were found in leaf tissue, one isozyme was associated with mesophyll cells, the other isozyme with bundle sheath cells (10). Biekmann and Feierabend (1) demonstrated that alanine aminotransferase activity in green leaves was predominantly localized in leaf microbodies and to a minor extent in the mitochondria. Lillo (15) has shown that the alanine aminotransferase activity undergoes diurnal fluctuations, as do several other enzymes.

We are interested in the regulation of genes in response to anaerobic stress. In anaerobic root tissue, an increase in ethanol and lactate is matched by an increase in the activity of ADH and LDH. Since under anaerobic conditions pyruvate is converted to alanine, we were interested in whether alanine aminotransferase activity also increased under anoxic conditions. We demonstrate that GPT activity does increase in activity in parallel with ADH activity. This increase in activity does not result in any change in GPT isozyme patterns as shown by native PAGE.

MATERIALS AND METHODS

Plant Material and Growth Conditions

Barley seeds (*Hordeum vulgare* L. cv Himalaya) were surface-sterilized in 1% NaOCl (w/v) for 20 min, rinsed with water, and planted in moist perlite. After 4 d of growth at 20°C, individual seedlings were removed from the perlite and inserted through a hole in a foam stopper. Plants in their

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[3] Abbreviations: ADH, alcohol dehydrogenase; GPT, alanine aminotransferase; LDH, lactate dehydrogenase; TEMED, *N,N,N',N'*-tetramethylethylenediamene.

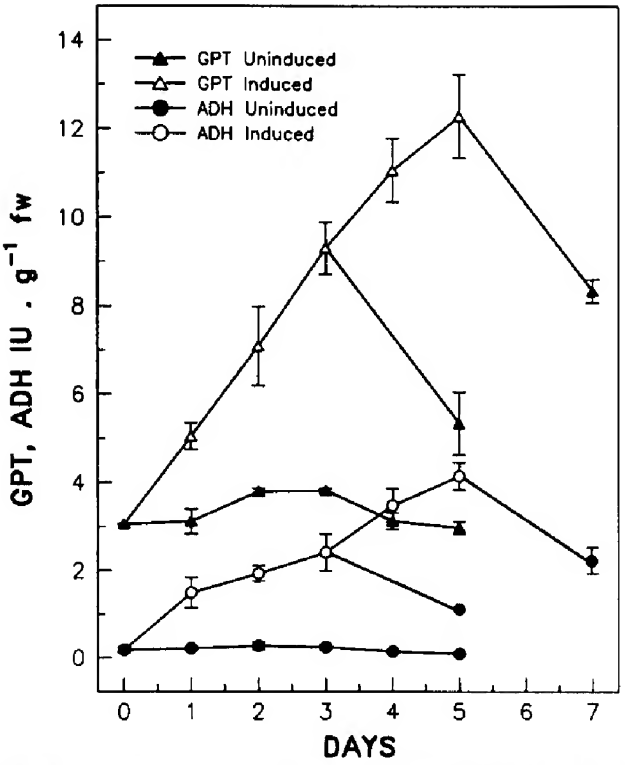

Figure 1. Induction of alanine aminotransferase (GPT) and alcohol dehydrogenase (ADH) activity in hypoxic barley roots. Plants were sparged with air until 2 weeks old; N_2 then replaced air for half of the plants. GPT and ADH activities are the means of three plants; bars represent the standard error. A portion of the plants that had been induced for 3 or 5 d was transferred to tanks sparged with air and tested for activity after 2 d. fw, fresh weight.

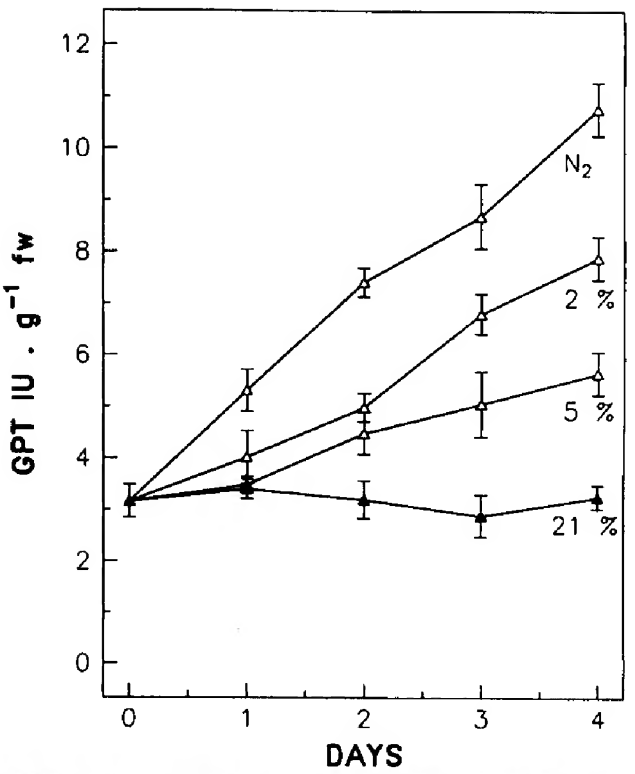

Figure 2. Induction of GPT activity under different O_2 concentration in the nutrient medium. Roots were sparged with various gas mixtures from premixed tanks and the O_2 levels in the nutrient solution monitored daily. Data points are the mean of three plants, bars represent the standard error. fw, fresh weight.

foam plugs were placed in a Plexiglas board cut to fit a 20-L fish tank. Plants were grown hydroponically with roots in darkness in half-strength Hoagland solution sparged continuously with air for 10 to 20 d. Hypoxic conditions were achieved by sparging the tank with N_2 or N_2 mixed with O_2. Growth chamber conditions were: day, 16 h, 20°C: night 8 h, 20°C; 250 μmol m^2 s^{-1} PPFD. Other cereals (*Zea mays* cv Spancross, *Triticum aestivum* cv Neepawa, *Secale cereale* cv Prima) were grown in the same way.

Extraction and Assay of ADH and GPT

Extractions were carried out at ice temperature. Roots were rinsed in distilled H_2O, briefly blotted, weighed, and ground with sand in a mortar and pestle in 0.1 M Tris-HCl (pH 8.0) containing 10 mM DTT, 15% (v/v) glycerol (EB). The brei was centrifuged for 3 min in a microcentrifuge and the supernatant assayed for enzyme activity. ADH assays were performed in the ethanol → acetaldehyde direction as previously described (8). GPT was assayed in the alanine → pyruvate direction by coupling the reaction to NADH reduction of lactate dehydrogenase as described by Lillo (15). The reaction mixture contained, in a final volume of 1 mL, 25 mM L-alanine, 5 mM 2-oxoglutarate, 0.1 mM NADH, 100 mM Tris-HCL (pH 8.0), 5 units of lactate dehydrogenase (Sigma L2375), and 50 μL of enzyme extract. The reaction was started by adding the 2-oxoglutarate and the assay temperature was 23°C. ADH and GPT activities are reported in international units (μmol/min).

Table I. *Induction of Alanine Aminotransferase Activity in Cereals*

All plants were sparged with air until 2 weeks of age. Roots were then sparged with air or N_2 for 5 d. N_2 leaf samples were from plants where the roots had been sparged with N_2.

	Root		Leaf	
	O_2	N_2	O_2	N_2
	IU/g fresh weight			
Maize	3.00 ± 0.46	6.84 ± 0.80	4.23 ± 0.81	3.75 ± 0.63
Barley	3.16 ± 0.37	11.97 ± 0.93	16.50 ± 1.19	14.37 ± 1.72
Rye	2.16 ± 0.24	7.56 ± 0.11	9.52 ± 1.49	6.58 ± 1.23
Wheat	3.09 ± 0.29	6.46 ± 0.86	17.52 ± 1.38	11.72 ± 0.82

Gel Electrophoresis

Nondenaturing electrophoresis was performed in slab gels (1.5 mm thickness) using 6% to 10% gradient gels. The 6% running gel buffer contained (w/v); 6.0% acrylamide-0.16% bisacrylamide, 5% (w/v) sucrose, and 0.37 M Tris-HCl (pH 8.9). The 10% running gel buffer contained (w/v); 10.0% acrylamide-0.26% bisacrylamide, 17% (w/v) sucrose, and

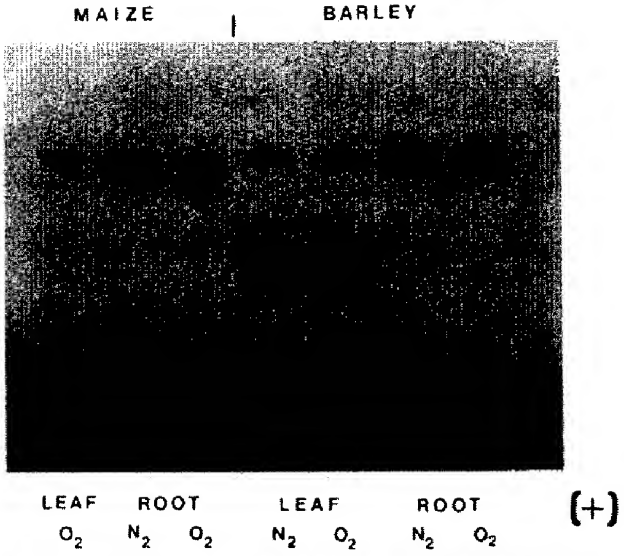

Figure 3. Native PAGE of cereal alanine aminotransferase activity. Samples are from uninduced (O_2) or induced (5 d N_2) root tissue. Leaf samples are also from uninduced or root induced (5 d N_2) plants. Each lane has the equivalent of 10 mg fresh weight of plant material loaded.

0.37 M Tris-HCl (pH 8.9). Both gel buffers were polymerized with 0.5 μL/mL TEMED and 0.32 mg/mL ammonium persulfate, and the gel was then immediately poured with a gradient former and peristaltic pump. The stacking gel was 5.0% acrylamide-0.13% bisacrylamide, 20% (w/v) sucrose, 60 mM Tris-HCl (pH 6.7), 0.6 μL/mL TEMED, and 5 μg/mL riboflavin, photo-polymerized for at least 3 h. Samples were homogenized in 10 mL of buffer/g fresh weight in EB. Typically, 90 μL of supernatant plus 10 μL of 90% glycerol containing 0.01% bromphenol blue was loaded. Gels were run at 4°C overnight. Gels were stained for GPT activity as described by Hatch and Mau (10). Briefly, gels were incubated for 30 min at 0°C in 50 mM Tris-HCl (pH 7.5) containing 20 units/mL LDH (Sigma). After warming to RT, the mixture was supplemented to 30 mM alanine, 10 mM 2-oxoglutarate, and 2 mM NADH. GPT activity from oxidized NADH resulted in nonfluorescent bands on a fluorescent background when viewed in UV light.

Nitrate-Free Nutrient Solution

Hoagland nutrient solution was prepared as described (23). The nitrate-free nutrient solution contained 4 mM $CaCl_2$, 2 mM $MgSO_4 \cdot 7H_2O$, 6 mM KCl, 1 mM $NaH_2PO_4 \cdot H_2O$, 0.07 g/L sequestrene 330 Fe, and 0.1 mL/L of the standard Hoagland micronutrient solution (21). The pH was then adjusted to 6.7 with NaOH.

RESULTS

Induction of GPT Activity

Alanine aminotransferase activity increased approximately fourfold after 5 d of induction (Fig. 1). No further induction in GPT activity was observed after that time. This increase in

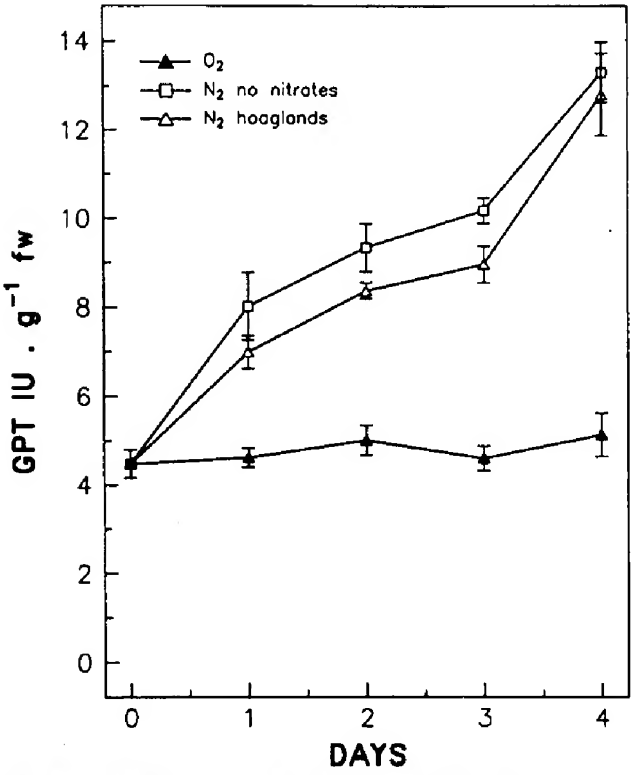

Figure 4. Induction of GPT activity in Hoagland solution and a N-free nutrient solution ("Materials and Methods"). Plants were sparged with air until 2 weeks old, then N_2 replaced air. The control plants were grown in Hoagland solution sparged with air. GPT activity are the means of three plants; bars represent the standard error. fw, fresh weight.

GPT activity paralleled the increase in ADH activity in the same root tissue; however, GPT activity was at least fourfold higher than the ADH activity (Fig. 1). It was necessary to maintain continuous hypoxia to retain the high level of GPT activity. When barley roots were returned to aerobic conditions, the level of GPT activity declined with an apparent half-life of 2 d. This decline in GPT activity was matched by a decline in ADH activity (Fig. 1) and is similar to what has been found for LDH (11).

Figure 2 demonstrates that GPT activity in barley roots is correlated with the degree of O_2 deficiency; the lower the level of O_2, the higher the level of GPT activity. Similar results have been found for the induction of LDH in barley root tissue (11). The induction of GPT activity also occurred in the roots of maize, wheat, and rye (Table I); however, the induction of barley GPT activity levels were higher than in any of the other three species. The level of GPT activity in leaf tissue was higher than in uninduced root tissue; however, anaerobic treatment of root tissue did not result in any increase in leaf GPT activity (Table I). Instead, plants with anoxic roots had lower levels of GPT activity in their leaf tissue.

Isozyme Profile of GPT

The isozyme profile of GPT is shown in Figure 3. Root tissue from either induced or uninduced barley plants had a

single band of GPT activity. The increase in root specific GPT activity resulted from an increase in activity of this single band; there was no evidence that, under hypoxia, different isozymes were expressed in comparison to aerobic conditions. In contrast, barley leaf tissue displayed two bands of activity, with the second band having much greater mobility than the root activity. Maize root and leaf tissue displayed three bands of activity, the bands with greater mobility having much lower levels of activity (Fig. 3). There was no change in the GPT activity pattern of maize root or leaf tissue when the root tissue was under anaerobic conditions.

GPT Induction under Low Nitrate Conditions

Hoffman *et al.* (11) have pointed out that the accumulation of alanine in hypoxic root tissue may depend on adequate N nutrition. The barley plants grown in these experiments were supplied with 7.5 mM NO_3^- in the nutrient solution. To test whether the induction of alanine aminotransferase was influenced by the level of NO_3^- in the nutrient solution we transferred plants to a nitrogen-free nutrient solution (see "Materials and Methods",) for 24 h, and then sparged plants with N_2 for 5 d. The data shown in Figure 4 demonstrate that the level of GPT activity increased similarly in Hoagland solution and in a N-free nutrient solution.

DISCUSSION

Alanine aminotransferase activity has been characterized in the leaves and cellular components of a number of different plant species (1, 9, 10). It is involved in alanine biosynthesis, and different GPT isozymes have been shown to be associated with the mesophyll and bundle sheath cells in several C_4 pathway plants (10). The observation that high levels of GPT activity compared with ADH or LDH activity were found in uninduced roots was not surprising, since GPT has functions in the plant cell other than just fermentative metabolism. However, the demonstration that GPT activity increases during hypoxia in a pattern similar to ADH indicates that this enzyme also undergoes a similar type of anaerobic induction (Fig. 1). This similarity in pattern of induction is further emphasized by the fact that lower levels of O_2 resulted in higher GPT activity (Fig. 2).

In contrast to ADH, where different genes are induced under anaerobic conditions (6, 8, 18), the induction of GPT activity in root tissue did not result in the differential increase in any specific band of GPT activity. Barley and maize root tissue and maize leaf were characterized by bands of activity that migrate very close together on native PAGE gels. In contrast, barley leaf material expressed a second band of activity of much higher mobility than found in root tissues (Fig. 3). Hatch and Mau (10) have shown that there are two GPT isozymes in a number of C_4 plants and that the isozyme present in bundle sheath cells corresponds to the higher mobility band. Surprisingly, we found that barley (a C_3 plant) had a pattern of GPT activity more akin to Hatch and Mau's (10) observations than did maize, a C_4 plant. The induction of GPT activity in all four species studied indicates that this induction is a general phenomenon found in the Gramineae. However, the levels of induction of this enzyme differ, as has been observed for LDH (11). In barley the induction of ADH activity in the root tissue results in an increase in ADH acrtivity in leaf tissue (16). However, GPT activity in leaf tissue did not increase when the plants roots were under anaerobic conditions. In fact, GPT activity levels were slightly lower under these conditions.

The large fluxes of ^{14}C to alanine from ^{14}C sugars (11, 21) indicate that large amounts of alanine may accumulate in anaerobic roots. However, under normal ecological conditions, one of the results of soil hypoxia is denitrification (5). We found that GPT activity is induced in both Hoagland solution and a N-free nutrient solution. Therefore, the induction of this enzyme is not dependent on the level of N nutrition during prolonged hypoxia. However, whereas GPT activity is induced in hypoxic roots under conditions of low N, low levels of glutamate (the amino donor) could reduce the levels of alanine production.

Our results demonstrate that cereal roots possess an alanine aminotransferase activity that is induced under anaerobic conditions similar to ADH and LDH induction. Previous work (11, 21) has demonstrated that ethanol glycolysis and alanine synthesis are major pathways of pyruvate metabolism in anaerobic root tissue. However, unlike ADH and LDH, the production of alanine by GPT does not regenerate NADH. Why, then, would root tissue produce such large amounts of alanine under anaerobic conditions? In contrast to ethanol, which diffuses out into the media, most of the alanine produced under anaerobic conditions is retained in the root tissue (11). Sakano and Tazawa (19, 20) have pointed out that the free amino acids in the vacuole of plant cells are relatively inactive in the metabolic sense and contribute to cytoplasmic homeostasis. However, during times of deficiency, these vacuolar amino acids can be transported across the tonoplast membrane into the cytoplasm and then utilized. They observed that alanine is the most rapidly transported amino acid (20). Perhaps under anaerobic conditions alanine is produced in large quantities but stored in the vacuole, where it is metabolically inactive until such time as it can be utilized by the cell. In this way the cell would retain the pyruvate carbons that would be lost if the cell were to produce ethanol which could diffuse out into the media. Although the level of GPT activity in anoxic root tissue suggests that alanine formation may be a major pathway in fermentative metabolism, the levels of substrates may limit the production of alanine in N-limited conditions. Further studies along this line are currently in progress in our laboratory.

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Exhibit 7

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Biochemistry

Feedback inhibition of fully unadenylylated glutamine synthetase from *Salmonella typhimurium* by glycine, alanine, and serine

(x-ray crystallography/protein structure/effector binding)

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ABSTRACT **Bacterial glutamine synthetase (GS; EC 6.3.1.2) was previously shown to be inhibited by nine end products of glutamine metabolism. Here we present four crystal structures of GS, complexed with the substrate Glu and with each of three feedback inhibitors. The GS of the present study is from *Salmonella typhimurium*, with Mn^{2+} ions bound, and is fully unadenylylated. From Fourier difference maps, we find that L-serine, L-alanine, and glycine bind at the site of the substrate L-glutamate. In our model, these four amino acids bind with the atoms they share in common (the "main chain" $^{+}NH_3$-CH-COO^{-}) in the same positions. Thus on the basis of our x-ray work, glycine, alanine, and serine appear to inhibit GS-Mn by competing with the substrate glutamate for the active site.**

Glutamine synthetase (GS) catalyzes the formation of glutamine from glutamate and ammonia with the concomitant hydrolysis of ATP into ADP and P_i. The amide group of glutamine serves as a nitrogen source for the biosynthesis of nitrogen-containing metabolites (1, 2). The original kinetic studies by Woolfolk and Stadtman showed that *Escherichia coli* GS was inhibited by nine end products of glutamine metabolism: serine, alanine, glycine, AMP, CTP, tryptophan, histidine, carbamoyl phosphate, and glucosamine 6-phosphate (3–5). Each inhibitor was found to inhibit part of the GS activity, and collectively they were found almost to abolish GS activity. These findings led Woolfolk and Stadtman to propose "cumulative feedback inhibition." In their model each inhibitor binds to a separate site, distinct from the catalytic site, and each is independent in its action.

The question of the number of distinct effector binding sites on the surface of GS was studied intensively in the 1970s. In addition to kinetic measurements, methods including NMR, EPR, fluorimetry, equilibrium binding, and calorimetry were all applied to study this complicated feedback regulatory system. Studies of equilibrium binding and of calorimetric measurement of AMP, L-tryptophan, L-alanine, and L-glutamate suggested that these effectors bind at separate sites on GS (6–8). Fluorescence measurements and NMR led to the conclusions that low- to unadenylylated GS possesses allosteric sites for amino acid inhibitors and that GS has different sites for L-glutamate, L-alanine, D-alanine, and glycine (9).

Different conclusions were reached by Dahlquist and Purich (10), who examined the interaction of low- to unadenylylated GS with the eight feedback inhibitors by magnetic resonance techniques. Their results suggested that the feedback inhibitors alanine, tryptophan, histidine, and glycine bound to the glutamate substrate site. Also, according to Rhee *et al.* (11), titration and kinetic data suggested that glycine can bind to both the L-alanine and D-alanine sites. A common site for L-glutamate and L-alanine appeared when the binding of L-glutamate to GS was measured in the presence of ADP and P_i (6, 9). Citing these studies and unpublished data, Stadtman and Ginsburg (2) concluded "there are separate sites on the enzyme for alanine, tryptophan, histidine, AMP, and CTP, whereas mutually exclusive binding occurs between glycine, serine, and alanine" (P. Z. Smyrniotis and E. R. Stadtman, unpublished data cited in review reference 2). And Rhee *et al.* (11) reviewed further evidence for separate sites of inhibition. In short, feedback inhibition of GS is a complicated regulatory system, worthy of continued study.

X-ray crystallography is well suited for the definition of binding sites. By x-ray methods, an initial atomic model for the 5616 residues of dodecameric unadenylylated GS from *Salmonella typhimurium* was determined at 3.5-Å resolution by Almassy *et al.* (12) and refined by Yamashita *et al.* (13). Recently a 2.8-Å-resolution atomic model has been refined based on x-ray diffraction data collected from one GS crystal of fine quality. This native model† is used here with the Fourier difference method to define the interactions of L-alanine, L-serine, L-glutamate, and glycine with fully unadenylylated GS-Mn from *S. typhimurium*.

MATERIALS AND METHODS

Purification of GS. GS for crystallization was isolated by ammonium sulfate precipitation and Cibracon Blue affinity column (14), whereas the protein for kinetic measurements was purified by ammonium sulfate precipitation (5). In the former method, ATP was used to elute GS from the column and could not be removed completely by extensive dialysis due to its high affinity for the enzyme. The bound ATP on GS could conceivably affect the kinetic measurements and hence was avoided.

Crystal Soaking and Data Collection. Fully unadenylylated crystals of GS from *S. typhimurium* were grown by the hanging-drop method of vapor diffusion (15). An effector was dissolved in the synthetic mother liquor containing 15 mM imidazole/HCl (pH 7.0), 3 mM $MnCl_2$, 3 mM spermine tetrahydrochloride, and 10% 2-methyl-2,4-pentanediol (MPD). Since the sudden replacement of mother liquor with synthetic mother liquor degrades the diffraction quality of GS crystals, half the volume of real mother liquor (5–10 μl) was replaced by the synthetic mother liquor containing one effector. In this way, L-serine, L-alanine, and glycine were added to GS crystals and allowed to diffuse for at least 1 day. Estimated final concentrations of L-glutamate, L-serine, L-alanine, and glycine were 10 mM, 30 mM, 15 mM, and 15 mM, respectively. X-ray data of GS–inhibitor complex crystals (Table 1) were collected with an R-AXIS-II image plate

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Abbreviation: GS, glutamine synthetase.

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†The atomic coordinates and structure factors have been deposited in the Protein Data Bank, Chemistry Department, Brookhaven National Laboratory, Upton, NY 11973 (reference 1LGS).

Table 1. Summary of data collection of GS–effector complexes

Crystal	Resolution, Å	Unique/total reflections	R_{sym},* %	$\langle \Delta F \rangle / \langle F \rangle$,† %
GS	2.8	106,965/234,915	7.8	—
GS–Glu	2.8	110,184/170,218	6.0	17.8
GS–Ser	2.9	98,318/145,232	6.4	9.6
GS–Ala	2.8	93,308/147,344	7.9	12.0
GS–Gly	2.8	104,689/161,708	7.4	9.6
GS–Glu–Ala	2.9	90,491/136,568	6.4	16.6

*On intensity.
†Mean fractional isomorphous difference, $\Sigma || F_{PH}| - |F_P|| / \Sigma |F_P|$.

detector (Rigaku, Denko, Japan). All complex crystals were isomorphous with respect to the native GS crystals, having space group $C2$ and unit-cell dimensions a = 235.5 Å, b = 134.5 Å, c = 200.1 Å, and β = 102.8°.

Fourier Difference Maps, $F_{o(GS\text{–effector})} - F_{o(GS)}$. Fourier difference maps with a 12-fold average, using Fourier coefficients $[F_{o(GS\text{–effector})} - F_{o(GS)}]$, were calculated by using CCP4 programs (Science and Engineering Research Council Collaborative Computing Project no. 4, Daresbury Laboratory, Warrington, U.K.) implemented on a DEC VAX 4000 computer at the University of California, Los Angeles. Phases of the 2.8-Å native GS model were used as the initial phases of the complexes, as justified by the crystal isomorphism. The 12-fold averaged difference maps displayed little noise above the 1σ contour level.

GS Assay. The enzymatic activity of GS can be measured by the formation of P_i in the biosynthetic reaction (16) and by the formation of γ-glutamylhydroxamate in the glutamyl transfer reaction (17). In order to compare with the kinetic data of Woolfolk and Stadtman (5), kinetic measurements were performed under conditions which were virtually identical to their conditions.

For the biosynthetic assay, 80 μl of reaction mixture containing 100 mM imidazole/HCl (pH 7.0), 100 mM $MgCl_2$, 100 mM NH_4Cl, 20 mM ATP, and various concentrations of L-glutamate was mixed with 80 μl of GS at 37°C to initiate the reaction. After 9 min, the reaction was terminated by adding 640 μl of 1% $FeSO_4$ in 7.5 mM H_2SO_4; 10 sec later, a faint blue color was developed by the addition of 60 μl of 6.6% ammonium molybdate/3.75 M H_2SO_4. The P_i produced was measured from OD_{660} 1 min after ammonium molybdate was added.

For the transferase assay, 250 μl of reaction mixture containing 150 mM triethanolamine, 50 mM dimethyl glutarate, 300 mM KCl, 20 mM $KAsO_4$, 20 mM NH_2OH·HCl, 0.4 mM ADP, 0.4 mM $MnCl_2$, and various concentrations of L-glutamate were combined with 100 μl of GS at 37°C to start the reaction. After 10 min, the reaction was stopped by adding 1 ml of 3.3% $FeCl_3$/2% trichloroacetic acid/0.25 M HCl. The OD_{540} was read 3 min later to determine the amount of glutamylhydroxamate produced.

RESULTS

Model Building of Effectors into Fourier Difference Maps. Five 12-fold averaged Fourier difference maps are shown in Fig. 1. All the strongest peaks in these clear difference maps at the 2σ contour level appear at the same position in the active site. Atomic models of L-glutamate, glycine, L-alanine, and L-serine were built into the strongest density peaks in their respective difference maps (Fig. 1 *a–d*). The atomic model of glycine was built first because the density peak suggests the orientation of glycine. The main chain of L-glutamate was built at the same position as glycine because of the common atoms they have. The side chain of L-glutamate was built into difference density projecting toward the ATP binding site for the formation of γ-glutamyl phosphate in the biosynthetic reaction (2, 3). The atomic models of L-alanine and L-serine were built in their difference maps, based on the glycine model. The overlapping atomic models (Fig. 1*f*) constitute evidence that these feedback inhibitors bind to the main-chain region of the L-glutamate binding site.

In the difference map of GS–Ser, in addition to the strongest peak assigned L-serine as explained in the preceding paragraph, there is a positive density peak and a negative peak on opposite sides of Asn-264 (Fig. 1*g*). The positive peak cannot be the L-serine, because its size is too small to fit the atomic model of the effector and because it overlaps partially the side chain of Asn-264. The two peaks can, however, be interpreted as the motion of Asn-264 away from the serine site and toward the ε-amino group of Lys-176. This movement can relieve overlap of the amide group of Asn-264 with the α-amino group of serine (1.9 Å).

The positive peaks and the negative peaks in the difference map $[F_{o(GS\text{–}Ser)} - F_{o(GS)}]$ (Fig. 1*g*) along residues 322–329 show that this segment becomes more ordered upon serine binding. There is little electron density in the averaged $(2F_o - F_c)$ map of native GS but there is density in that of GS–Ser (Fig. 1*h*). The formation of one hydrogen bond between the γ-carboxylate group of Glu-327 and the β-hydroxyl group of serine might stabilize this segment. However, this segment does not seem to be similarly stabilized in the GS–Glu complex, perhaps because of the electrostatic repulsion between the substrate glutamate and the Glu-327 residue.

The density peak of L-serine in its difference map can be interpreted in terms of the model of Fig. 2. As judged by atom types and separation, the α-carboxylate group of L-serine forms hydrogen bonds with the guanidino group of Arg-321. Similarly, the α-amino group of L-serine forms a hydrogen bond with the carbonyl group of Gly-265 and a hydrogen bond with the γ-carboxylate group of Glu-131. The β-hydroxyl group of L-serine may form one hydrogen bond with the γ-carboxylate group of Glu-327. The interactions of L-alanine and glycine with GS are very similar to those of serine except that they lack the interactions of the β-hydroxyl group.

Kinetic Measurements of GS. To reconcile our structural studies with the earlier models for feedback control of GS, we investigated the inhibition of GS by glycine, L-alanine, and L-serine by both the biosynthetic assay and the glutamyltransferase assay (5). Our kinetic studies were carried out with the protocol of Woolfolk and Stadtman (5) with little modification. The only difference is that we used a smaller volume—0.16 ml of assay mixture and 0.64 ml of stop mixture instead of 0.2 ml and 1.8 ml, respectively—so that the blue color would be less diluted.

Studies by the biosynthetic assay (data not shown) present a complicated picture, but the transferase assay (Fig. 3) gives a simple result of competitive inhibition kinetics for glycine, L-alanine, and L-serine. With the biosynthetic assay, both K_m and V_{max} experience changes at low inhibitor concentrations ("uncompetitive"), and V_{max} is changed at higher inhibitor concentrations ("noncompetitive"). But with the transferase assay, only the apparent K_m changes in the presence of alanine, glycine, and serine (competitive inhibition). The estimated K_i values for alanine, glycine, and serine are 0.16, 0.8, and 1.1 mM, respectively. Also, when the data are plotted in single reciprocal form ([glutamine]/velocity vs. [glutamine] at various serine, alanine, and glycine concentrations) parallel lines are observed with slopes of 25.2 ± 0.6, suggesting competitive inhibition.

DISCUSSION

Interactions of Glycine, L-Alanine, and L-Serine with GS. Comparison of the structures of GS–Ser, GS–Ala, and GS–Gly complexes with that of the GS–Glu complex (Fig. 1*e*) presents direct evidence for the similarity in binding to GS of these inhibitors and of glutamate. Based on the difference maps, L-serine, L-alanine, and glycine appear to occupy essentially

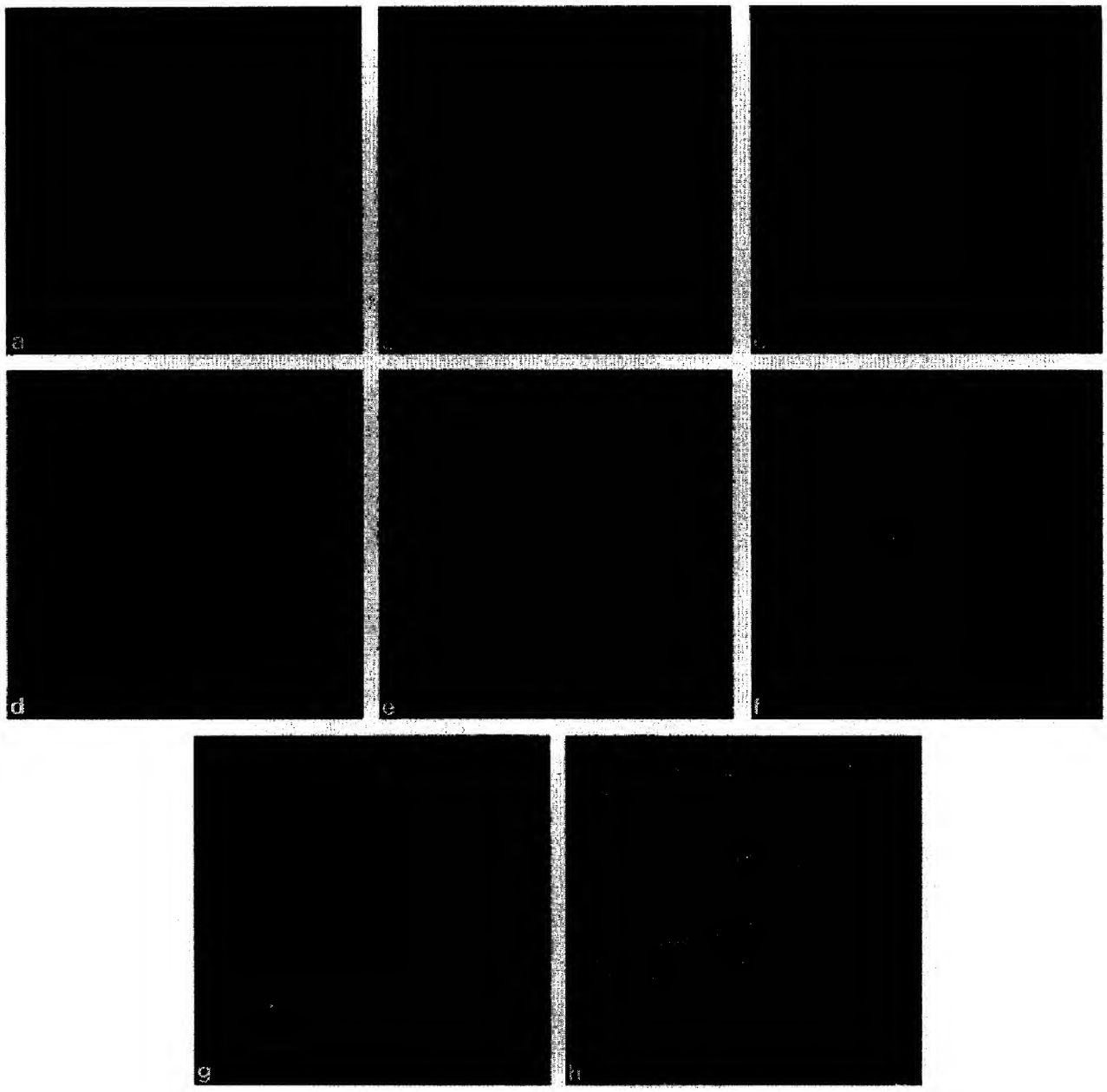

FIG. 1. The strongest peaks in the 12-fold averaged Fourier difference maps [$F_{o(GS\text{–effector})} - F_{o(GS)}$] before refinement. (*a*) $F_{o(GS\text{–Glu})} - F_{o(GS)}$. (*b*) $F_{o(GS\text{–Gly})} - F_{o(GS)}$. (*c*) $F_{o(GS\text{–Ala})} - F_{o(GS)}$. (*d*) $F_{o(GS\text{–Ser})} - F_{o(GS)}$. (*e*) $F_{o[GS\text{–(Glu+Ala)}]} - F_{o(GS)}$. (*f*) The atomic models: L-glutamate is shown in blue, L-serine in yellow, L-alanine in green, and glycine in red. These overlapping models suggest that L-glutamate, L-serine, L-alanine, and glycine occupy the same site for the atoms they have in common. (*g*) The difference map $F_{o(GS\text{–Ser})} - F_{o(GS)}$ in greater detail, but from a different point of view than *d*. The positive density map is shown in orange, the negative map in blue, and the GS atomic model in yellow. There are three positive density peaks at 1.2σ contour level; one is the serine peak with its atomic model; another, beside Asn-264, shows the movement of Asn-264 away from the serine site; the third implies that the segment 322–329 becomes more ordered. (*h*) $2F_o - F_c$ map of native GS is displayed in blue and that of the GS–Ser complex in red. In the native GS $2F_o - F_c$ map, virtually no density is observed in residues 326–327. However, the electron density for Tyr-327 and Glu-327 is seen in the map of the GS–Ser complex, indicating that these residues become more ordered upon serine binding.

the same site and might be expected to inhibit GS by competing for the active site against the substrate L-glutamate. This conclusion is consistent with the original notion of Woolfolk and Stadtman (5) about multiple feedback inhibition but suggests a mechanism for these inhibitors on GS–Mn which differ from theirs and from that of Rhee *et al.* (9). Our mechanism is consistent with the mechanism of Dahlquist and Purich (10) for alanine, glycine, and serine and with the mechanism based on the unpublished data of Smyrniotis and Stadtman cited in ref. 2. We note also that other authors were not studying fully unadenylylated GS–Mn from *S. typhimurium*, but rather *E. coli* GS in various states of adenylylation and with Mg^{2+} as well as Mn^{2+}. In a biochemical system as complicated as GS, these differences could conceivably account for some differences in effector action.

Kinetic Measurements of GS in the Presence of Glycine, L-Alanine, and L-Serine. The kinetic data from the transferase assay are consistent with inhibition of GS by L-alanine,

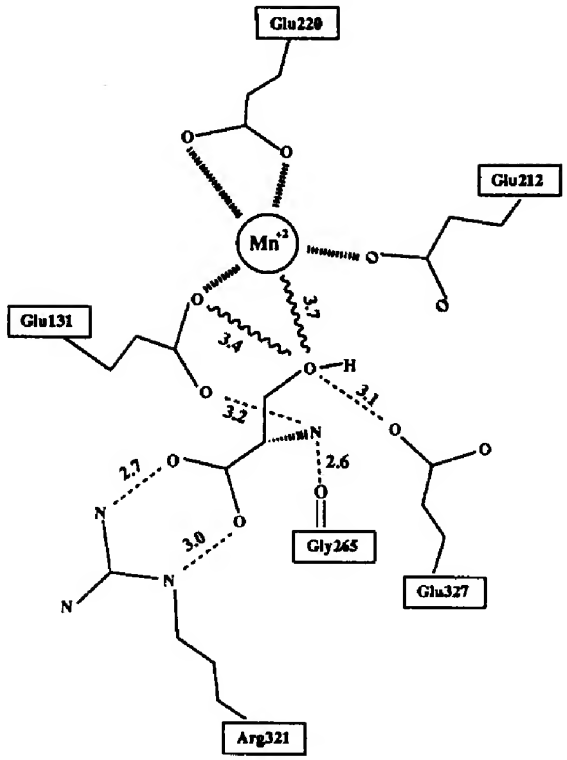

FIG. 2. Model for interactions of L-serine with GS as discussed in the text. Hydrogen bond lengths and other separations are shown by numbers (in angstroms). Glu-131, Glu-212, and Glu-220 are ligands for Mn^{2+}. It is uncertain that both oxygen atoms of Glu-220 coordinate to Mn^{2+}.

glycine, and L-serine by competition for the active site. The more complicated pattern of kinetics observed with the biosynthetic assay may be explained in two ways. The first explanation is metal dependence. The transferase assay uses GS–Mn whereas the biosynthetic assay uses GS–Mg, because completely unadenylylated GS from *S. typhimurium* and *E. coli* (18) has no biosynthetic activity in the presence of Mn^{2+}. Our crystal data were collected from GS–Mn, which presumably is the same GS–Mn form that carried out the transferase activity. However, Mg^{2+} and Mn^{2+} have markedly different effects on catalytic parameters and on the inhibitory response to different inhibitors (2, 18); thus they may stabilize different conformations of GS, and thus the biosynthetic assay may suggest a more complicated mode of catalysis for GS–Mg. Whether Mg^{2+} actually stabilizes a different conformation we will learn only when we determine the structure of GS–Mg.

The second explanation for the complicated behavior of the biosynthetic assay is the relative stabilities of complexes GS–ATP–Glu, GS–ADP–(P_i)–Glu, GS–ATP–inhibitor, and GS–ADP–(P_i)–inhibitor. The K_m or K_i values (19) and the dissociation constants (8, 9, 20–22) indicate that the binding affinities of ATP and ADP for GS are stronger than those of glutamate and other amino acids. Also, ATP is believed to be the first substrate to bind to GS (19). Thus glutamate, serine, alanine, and glycine are competing to bind to GS–ATP instead of GS. The kinetics of the biosynthetic reaction may indicate that glycine, alanine, and serine form more stable complexes with GS–ADP, and less stable with GS–ATP. If so, then in the transferase assay, competition is observed because both the substrate and the inhibitor bind to the same enzyme form, namely GS–ADP–P_i, or GS–ADP–AsO_4^-. However, if glycine, alanine, and serine do not bind as strongly to GS–ATP, a noncompetitive component to the

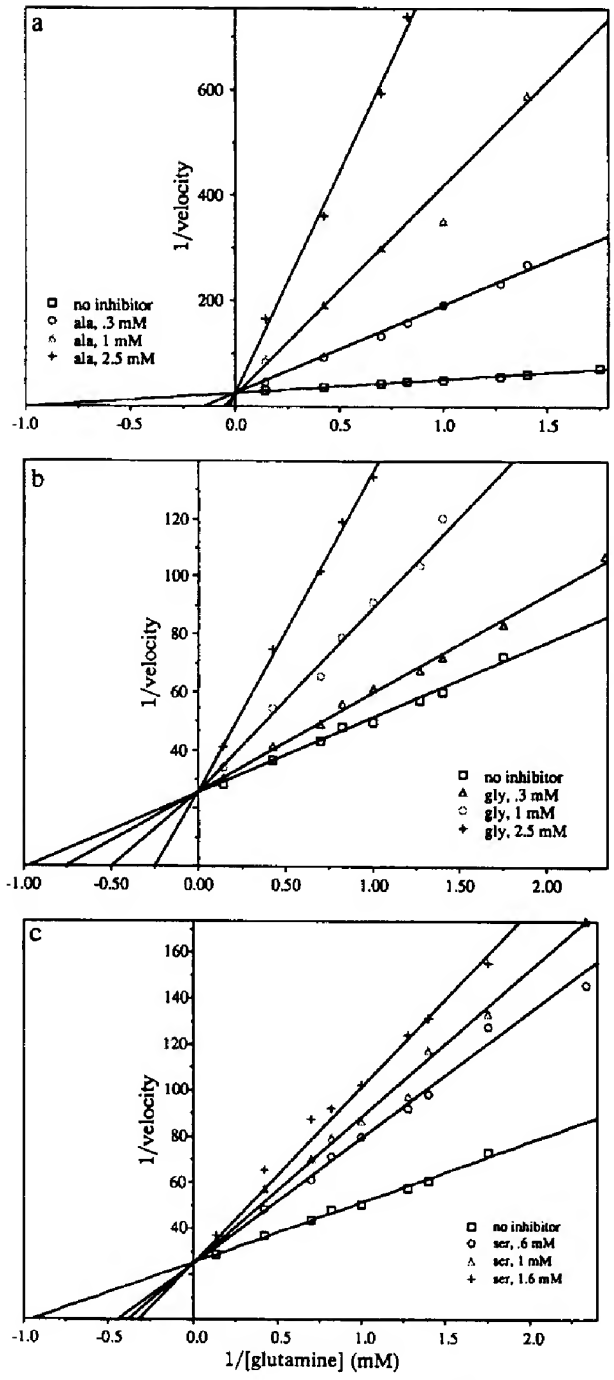

FIG. 3. Kinetic studies of L-alanine, L-serine, and glycine using the transferase assay for GS–Mn. Double-reciprocal plots are shown in which 1/velocity is plotted as a function of 1/[glutamine] in the presence of L-alanine (*a*), glycine (*b*), or L-serine (*c*). These kinetic data suggest that L-serine, L-alanine, and glycine are competitive inhibitors of GS–Mn with respect to L-glutamine.

inhibition will be observed. This pattern of a noncompetitive component to the kinetics of an enzyme that is inhibited at the active site was observed for the inhibition of alcohol dehydrogenase by auramine O (23). In that case, noncompetitive inhibition in the initial rate studies was explained by the ability of auramine O to form a ternary complex with enzyme–NAD^+ and enzyme–NADH binary complexes.

A noncompetitive component in the biosynthetic assay is supported by the dissociation constants (8, 9, 20–22). These

dissociation constants suggest that GS–Mg–ATP–Glu is more stable than GS–Mg–ADP–P_i–Glu, whereas GS–Mg–ATP–Ala is less stable than GS–Mg–ADP–P_i–Ala. Previous measurements also show that in the presence of GS–Mg–ADP, alanine enhances P_i binding 20-fold, whereas glutamate decreases P_i binding by a factor of 25 (8). These relative stabilities suggest that the noncompetitive component of the GS biosynthetic reaction in the presence of L-alanine may be consistent with inhibition of L-alanine at the GS active site, in analogy with the case of alcohol dehydrogenase (23). Further, the surprising observation of noncompetitive inhibition by the product L-glutamine in the biosynthetic reaction (19) could have a similar explanation.

The Fourier Difference Map $F_{o[GS-(Glu+Ala)]} - F_{o(GS)}$. The structures of the complexes GS–Gly, GS–Ser, and GS–Ala were determined in the absence of any substrate. Could these inhibitors bind to a second site in the presence of glutamate? The crystal structure of GS–Mn soaked with both 10 mM L-glutamate and 15 mM L-alanine has been determined in order to investigate the existence of a second binding site or to confirm the competitive inhibition mode between glutamate and alanine. According to our kinetic data, about 60% of the biosynthetic activity of GS–Mg in the presence of 10 mM L-glutamate is lost due to the inhibition by 15 mM L-alanine. As presented in Fig. 1 *a* and *c*, a peak is observed in the difference map of GS–Glu (10 mM L-glutamate), and a peak in the same site is also found in that of GS–Ala in the presence of 15 mM L-alanine.

In the difference map $\{F_{o[GS-(Glu+Ala)]} - F_{o(GS)}\}$, the strongest peak appears at the main-chain region of the L-glutamate binding site (Fig. 1*e*). No other density peak for a second binding site is observed. The size of the strongest peak is well fit by the atomic model of L-alanine. Also, density for the side chain of L-glutamate is not observed. This map suggests that most of the glutamate sites are occupied by alanine, not by glutamate. Thus this result is consistent with our hypothesis that L-alanine is a competitive inhibitor with respect to glutamate and its affinity for GS is not substantially lower than that of glutamate.

Is There a Conformational Change upon Inhibitor Binding? Further evidence supporting classical competitive inhibition for GS is provided by an analysis of quaternary and tertiary changes. There is no significant conformational change induced by the binding of these three inhibitors other than the movement of Asn-264. The large-scale structural changes in the dodecamer or within a monomer which are expected during an allosteric transition upon the binding of the inhibitor are not observed in these complexes. Of course, only small quaternary and tertiary changes were expected from crystal-soaking experiments because inhibitor soaks did not dissolve or crack GS crystals.

The movement of Asn-264 observed in the complexes GS–Glu, GS–Gln, GS–methionine sulfoximine, GS–Ser, GS–Ala, and GS–Gly could also support the common site for these effectors because, in our model, the amide group of Asn-264 collides with the α-amino group of these effectors. Moreover, engineered replacement of Arg-321 may provide further evidence for the common site, since the formation of hydrogen bonds between Arg-321 and these effectors may be important for their binding to GS. The replacement of Arg-321 by other amino acids (except possibly lysine) should result in lower binding of the substrate and of these inhibitors to GS.

Conclusion. Based on the crystal structures of GS–Ser, GS–Ala, GS–Gly, GS–Glu, and GS–(Glu+Ala) reported here, L-serine, L-alanine, and glycine bind to the L-glutamate site on unadenylylated GS–Mn from *S. typhimurium*, where they inhibit by competing for the substrate site. GS is regulated by multiple feedback inhibition but perhaps by a somewhat simpler mechanism than that proposed by Woolfolk and Stadtman (5).

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Exhibit 8

Plant Physiol. (1975) 55, 968–974

Pea Leaf Glutamine Synthetase

REGULATORY PROPERTIES[1]

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ABSTRACT

Of a variety of purine and pyrimidine nucleotides tested, only ADP and 5'AMP significantly inhibited the Mg^{2+}-dependent activity of pea leaf glutamine synthetase. They were less effective inhibitors where Mn^{2+} replaced Mg^{2+}. They were competitive inhibitors with respect to ATP, with inhibition constant (Ki) values of 1.2 and 1.8 mM, respectively. The energy charge significantly affects the activity of glutamine synthetase, especially with Mg^{2+}. Of a variety of amino acids tested, L-histidine and L-ornithine were the most inhibitory, but significant inhibition was seen only where Mn^{2+} was present. Both amino acids appeared to compete with L-glutamate, and the Ki values were 1.9 mM for L-histidine (pH 6.2) and 7.8 mM for L-ornithine (pH 6.2). L-Alanine, glycine, and L-serine caused slight inhibition (Mn^{2+}-dependent activity) and were not competitive with ATP or L-glutamate.

Carbamyl phosphate was an effective inhibitor only when Mn^{2+} was present, and did not compete with substrates. Inorganic phosphate and pyrophosphate caused significant inhibition of the Mg^{2+}-dependent activity.

Since glutamine synthetase (EC 6.3.1.2) catalyzes the first reaction in the complex branched pathways leading to the synthesis of a number of basic products, such as tryptophan, histidine, CAP[3], purine nucleotides, pyridine nucleotides, and glycine, serine, and alanine (5, 6), it was considered likely that it would prove to be a regulatory enzyme, as has been shown to be the case for many other enzymes occupying similar positions in other branched pathways (20).

Glutamine synthetase of a number of microorganisms is subject to feedback by a variety of end products, although there is considerable variation between species or genera with regard to the most effective compounds. When two or more inhibitors were added simultaneously, cumulative inhibition was seen in many of the microorganisms (6). This work has been extended considerably with the *Escherichia coli* enzyme (24) which is apparently unique in that it exists in two interconvertible forms, the interconversion of which depends on an additional enzyme and a regulatory protein, the latter which itself exists in two forms, catalyzed by two other enzymes (18). The *Bacillus subtilis* (4) and *Neurospora crassa* enzymes (8) have also been the subject of regulatory studies. The rat liver and ovine brain glutamine synthetases are also subject to feedback inhibition by the same compounds effective with the bacterial sources (21, 22). Here, as with the bacterial glutamine synthetase, most inhibitors were more effective if Mn^{2+} replaced Mg^{2+} in the assay medium.

Little information on the regulatory characteristics of higher plant glutamine synthetase was available until recently. Varner and Webster (23) found that the pea seed glutamine synthetase was inhibited by ADP and Pi. Caldos (2) reported that the purified carrot enzyme was slightly inhibited by Pi and a variety of purine and pyrimidine nucleotides, as well as by alanine, glycine, serine, and CAP.

More recently, Kanamori and Matsumoto (7) found that the partially purified rice root glutamine synthetase was inhibited by several purine nucleotides, but amino acids had no effect.

We recently reported the purification of glutamine synthetase (to apparent homogeneity) from pea leaves, and a number of its properties were described (13, 15). The present paper presents the results of studies dealing with the *in vitro* regulation of this enzyme by feedback inhibition.

MATERIALS AND METHODS

The enzyme was purified to at least 90% homogeneity (as judged by disc gel electrophoresis) from 10 to 20-day-old Blue Bantam pea shoots, as previously described (13). The enzyme was stored for periods of 1 to 60 days before use, in the presence of $MgSO_4$ (10 to 15 mM) and either 1 M sucrose or ethylene glycol (20 to 30%, v/v) (13). It was diluted considerably before assay, so that the final Mg^{2+} content during assay (derived from the enzyme) was below 0.1 mM in the cases where the Mn^{2+} biosynthetic assay was used. In some experiments the Mg^{2+} was removed by gel filtration on G-25 Sephadex prior to assay, or by dialysis against 25% ethylene glycol. The enzyme was assayed by procedures described previously (13, 15).

The amino acids and analogues, purine and pyrimidine nucleotides, and carbamyl phosphate were obtained from Sigma Chemical Co. The purine and pyrimidine nucleotides were generally the best grade available.

RESULTS

Inhibition by Purine and Pyrimidine Nucleotides. Since both purine nucleotides and pyrimidine nucleotides require L-glutamine for their synthesis, both classes of compounds can be regarded as end products. These were tested with both Mn^{2+} and Mg^{2+} as the cation sources in the reaction. Only three of a variety of purine and pyrimidine nucleotides had any significant effect on the enzyme activity.

[1] This work was supported by a grant from the National Research Council of Canada.

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[3] Abbreviation: CAP: carbamyl phosphate.

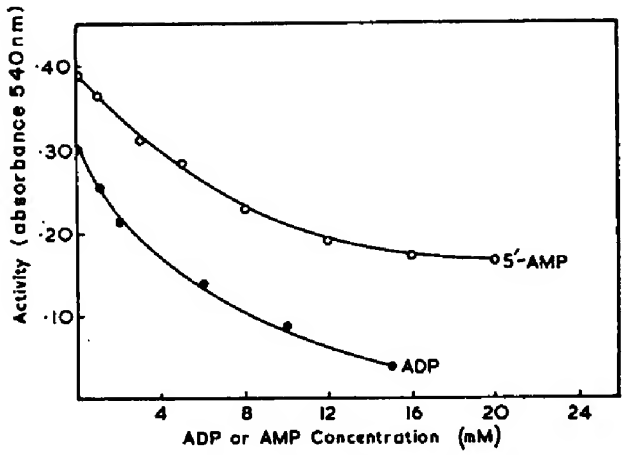

FIG. 1. Effect of ADP and 5'-AMP on Mg^{2+}-dependent glutamine synthetase activity. The reaction contained 6 mM ATP, 20 mM $MgSO_4$, 8 mM NH_2OH, 50 mM L-glutamate, 1 mM DTPA, and 0.1 M Tricine-KOH buffer, pH 7.8. Glutamyl hydroxamate synthesis was assayed.

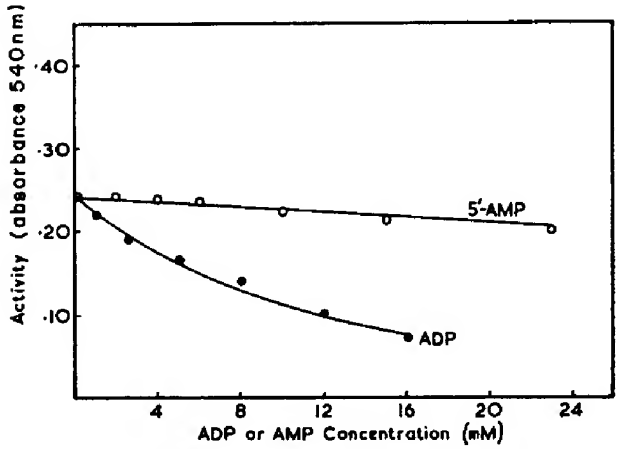

FIG. 2. Effect of ADP and 5'-AMP on Mn^{2+}-dependent glutamine synthetase activity. The reaction contained 6 mM ATP, 8 mM NH_2OH, 30 mM L-glutamate, 3 mM $MnSO_4$ and 0.1 M MES-KOH buffer, pH 6.2. Glutamyl hydroxamate synthesis was assayed. The results are from two separate experiments.

Compounds having no effect (when present equimolar with ATP, or even in 2-fold excess, or with limiting or nonlimiting L-glutamate) included GMP, UMP, CMP, IMP, UDP, GTP, ITP, CTP, and UTP. Adenosine was slightly inhibitory. The three nucleotides which did inhibit were ADP and 5'- and 2'-AMP. Some results with these inhibitors are shown in Figure 1 (2'-AMP caused only two-thirds as much inhibition as 5'-AMP and was not studied further). It was determined that ADP was a competitive inhibitor with respect to ATP and noncompetitive with respect to L-glutamate. Likewise, 5'-AMP was competitive with respect to ATP. The *Ki* values of ADP and 5'-AMP were approximately 1.2 mM and 1.8 mM, respectively.

Where Mn^{2+} was the divalent cation source it was found that 5'-UMP, 5'-IMP, and 5'-CMP caused 7 to 17% stimulation (at 4.6 mM, equimolar with ATP), while adenosine, ITP, UTP, and GTP caused 12%, 14%, 20%, and 5% inhibition, respectively. It is not surprising that ITP, UTP, and GTP cause such limited inhibition, in that they are capable of partially or nearly completely substituting for ATP where Mn^{2+} is the divalent cation (15). ADP and 5'-AMP were not as inhibitory with Mn^{2+} as they were with Mg^{2+} (Fig. 2).

Since ADP and 5'-AMP were inhibitory, it was expected that pea leaf glutamine synthetase would be significantly affected by the "energy charge" (1) as shown in Figure 3. The effect of a decrease in energy charge is more pronounced with Mg^{2+} than with Mn^{2+}. Only a small part of the decrease in activity (with either Mg^{2+} or Mn^{2+}) as the energy charge falls is caused by a reduction of ATP concentration, for the ATP concentrations at energy charges of 0.825 and 0.6 are, respectively, 5.63 mM and 3.29 mM, or 12.5 and 7.3 times the *Km* for ATP under these conditions (15).

Inhibition by Amino Acids. As mentioned earlier, several amino acids can be looked upon as end products whose synthesis requires glutamine. A variety of amino acids were tested to see if they would inhibit the enzyme. The results (in the presence of Mg^{2+}) are summarized in Table I. The amino acids caused only slight inhibition in most cases, even where L-glutamate was limiting. Where Co^{2+} was the divalent cation, the degree of inhibition was either greater or smaller, depending on the amino acid (Table II). It was less with L-alanine and its analogs and serine, but much more with L- and D-histidine. In the case of D-histidine, a brownish-orange color appeared in the assay tubes, indicating the probability of a cobalt-histidine complex, although this did not occur with an equal concentra-

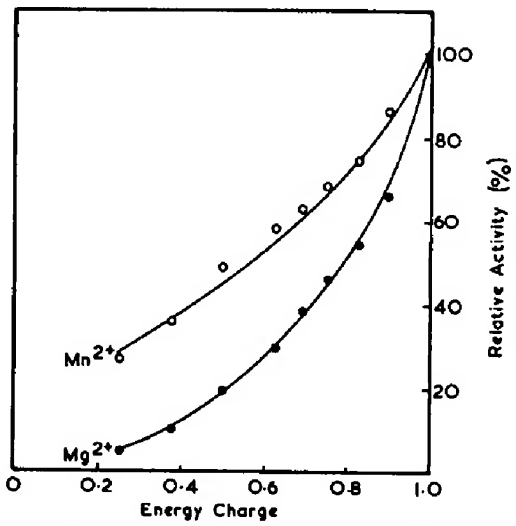

FIG. 3. Effect of energy charge on Mg^{2+}-dependent and Mn^{2+}-dependent glutamine synthetase activity. The desired energy charge was obtained using myokinase by the method of Klugsoyr *et al.* (9). In the Mg^{2+}-dependent assay, 20 μg (16 units) of myokinase was incubated 30 min at 35 C in the presence of the normal glutamine synthetase reaction components (100 mM L-glutamate, Tricine-KOH, pH 7.8, 3 mM NH_4Cl, 1 mM DTPA, and 20 mM $MgSO_4$) plus 8 mM total (ATP plus AMP) in a total volume of 1 ml. The reaction was then started with 0.1 ml of purified glutamine synthetase enzyme and was run for 5 min at 35 C. The modified Boyer Pi assay was employed (13). In the case of the Mn^{2+}-dependent assay, myokinase (20 μg) plus 8 mM (ATP plus AMP) was first incubated for 30 min at 35 C in the presence of 10.8 mM $MgSO_4$ and 0.03 M Tricine-KOH buffer, pH 8.1, in a total volume of 0.25 ml. Then 0.65 ml of glutamine synthetase substrates and buffer was added (0.15 M succinate-KOH buffer, pH 5.2, 60 mM L-glutamate, 6 mM NH_4Cl, and 12 mM $MnSO_4$) and the reaction started with 0.10 ml of purified glutamine synthetase. The reaction was terminated 5 min later and Pi assayed by the modified Boyer technique (13).

Table I. *Effect of Amino Acids on the Mg^{2+}-dependent Activity of Glutamine Synthetase*

The production of glutamyl hydroxamate was assayed. In part A the reaction contained 8 mM ATP, 20 mM $MgSO_4$, 1 mM diethylenetriamine pentaacetic acid, 10 mM mercaptoethanol, 12 mM L-Glu, 8 mM NH_2OH, pH 7.8. In part B the only difference was a higher concentration (36 mM) of L-glutamate.

Treatment	Relative Activity
mM	%
A. Control	100
D-Ala (12)	69
β-Ala (12)	88
L-Ala (12)	86
D-His (12)	92
L-His (12)	86
B. Control	100
Gly (12)	93
L-Arg (12)	94
L-Orn (12)	92
L-Tryptophan (12)	98
L-Citrulline (12)	96
L-Lys (12)	100

Table II. *Effect of Amino Acids on Co^{2+}-dependent Activity of Glutamine Synthetase*

In part A the reaction contained 6 mM ATP, 12 mM L-Glu, 10 mM $CoCl_2$, 1 mM diethylenetriamine pentaacetic acid, 8 mM NH_2OH, pH 6.7. In part B the glutamate concentration was 36 mM. The production of glutamyl hydroxamate was assayed.

Treatment	Relative Activity
mM	%
A. Control	100
D-Ala (12)	78
β-Ala (12)	91
L-Ala (12)	90
D-His (12)	45
L-His (12)	12
L-Tryptophan (12)	88
B. Control	100
L-Ala (12)	94
L-Ser (12)	99
L-Arg (12)	104
L-Citrulline (12)	104
L-Orn (12)	93

tion of L-histidine. Furthermore, increasing the cobalt level (L-histidine, 12 mM, ATP, 6 mM) from 12 mM to 14 mM to 16 mM increased inhibition from 69% to 73% to 76%, respectively, so the inhibition by L-histidine was not due to its chelation of cobalt.

Where Mn^{2+} was employed as the cation source, results were different again. As shown in Table III, L- and D-histidine were not as inhibitory as they were with Co^{2+}, but much more so than with Mg^{2+}. L-Alanine and its analogs were more inhibitory with Mn^{2+} than with either Co^{2+} or Mg^{2+}, as was L-ornithine. In an experiment designed to determine whether the L-histidine inhibition was caused by Mn^{2+} chelation, it was clear that increasing the Mn^{2+} level decreased the per cent inhibition by histidine, but the control activity was also decreased (Fig. 4). The decreased activity in either the absence or presence of L-histidine as Mn^{2+} (total) rises from 3 or 5 mM to 7 to 9 mM is caused by a pronounced shift in pH optimum, which varies considerably with the Mn^{2+} concentration (13). The fact that the decrease in activity with increasing Mn^{2+} is less pronounced

Table III. *Effect of Amino Acids on Mn^{2+}-dependent Activity of Glutamine Synthetase*

The production of glutamyl hydroxamate was assayed. The reaction contained: (part A) 6 mM ATP, 4 mM $MnSO_4$, 8 mM NH_2OH, 4 mM mercaptoethanol, 12 mM L-Glu, pH 6.2; (part B) 8 mM ATP, 4 mM $MnSO_4$, 8 mM NH_2OH, 12 mM L-Glu, pH 6.1; (part C) 6 mM ATP, 3.5 mM $MnSO_4$, 5 mM NH_4Cl, 10 mM L-Glu, pH 6.1.

Treatment	Relative Activity
mM	%
A. Control	100
L-Ala (12)	77
D-Ala (12)	46
β-Ala (12)	72
L-Tryptophan (12)	106
L-His (12)	44
D-His (12)	67
L-Lys (20)	100
B. Control	100
Glyc (12)	82
L-Ser (12)	81
L-Arg (12) or L-citrulline (12)	104
L-Orn (12)	64
C. Control	100
L-His (12)	39
L-3 Methylhistidine (12)	13
L-1 Methylhistidine (12)	70
Aminotriazole (12)	62
Histamine (12)	98
Imidazole acetic acid (12)	94

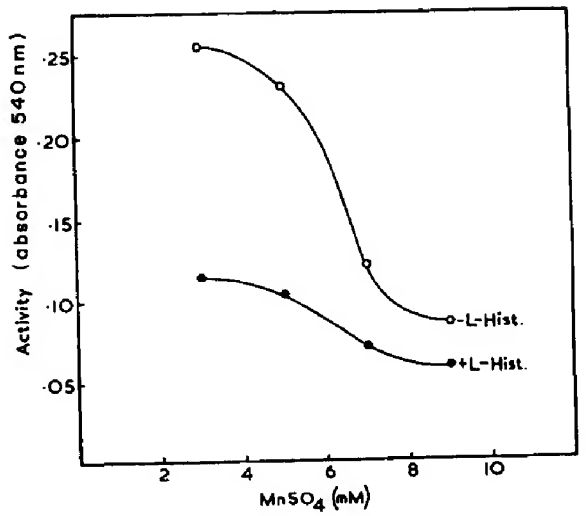

FIG. 4. Effect of $MnSO_4$ concentration on the inhibition of L-histidine. The reaction contained 6 mM ATP, 8 mM NH_2OH, 12 mM L-glutamate, and 0.1 M MES-KOH buffer, pH 6.2. Plus 12 mM L-histidine (●); minus L-histidine (○).

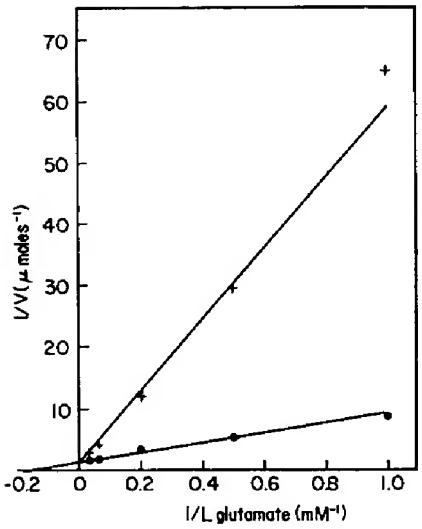

FIG. 5. Inhibition of Mn^{2+}-activatied glutamine synthetase by L-histidine. The reaction contained 6 mM ATP, 8 mM NH_4OH, 4 mM $MnSO_4$, and 0.1 M MES-KOH buffer, pH 6.2. Plus 10 mM L-histidine (+); minus histidine (⊕). Glutamyl hydroxamate synthesis was assayed.

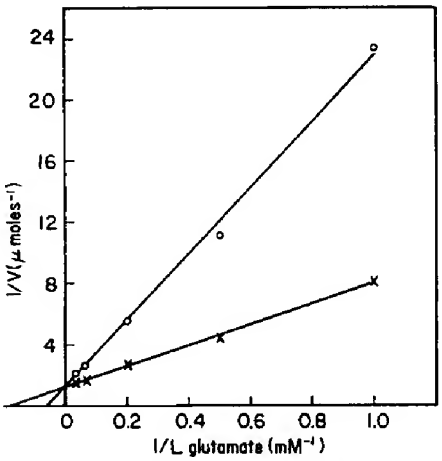

FIG. 6. Inhibition of Mn^{2+}-activated glutamine synthetase by L-ornithine. The reaction contained the same components as in Fig. 5, except ornithine replaced histidine. Minus ornithine (×); plus 20 mM L-ornithine (○).

in the presence of histidine may be due to the complexing of some of the excess Mn^{2+} by L-histidine.

L-Histidine and L-ornithine were competitive inhibitors with respect to L-glutamate, the substrate (Figs. 5 and 6) while L-alanine, glycine, and L-serine were uncompetitive inhibitors with respect to L-glutamate or NH_4Cl (Fig. 7). The inhibition caused by L-histidine was greater at pH 6.1 or 6.2 than it was at pH 5.2, but this may reflect the different ATP and Mn^{2+} concentrations used at the two pH values. The *Ki* value for L-histidine at pH 6.2 (ATP, 6 mM, Mn^{2+}, 3 mM) and pH 5.2 (ATP, 8 mM, Mn^{2+}, 11 mM) were 1.7 to 2.1 mM and 5.9 to 6.5 mM, respectively, while the *Km* values for L-glutamate in the same assays were 6 to 8.7 mM and 7.1 to 8.7 mM, respectively. The *Ki* for L-ornithine at pH 6.2 was 7.8 mM.

L-Histidine inhibition was approximately equal using either NH_4OH or NH_4Cl and using the Pi assay or the glutamyl hydroxamate assay.

A number of analogs of L-histidine were tested to compare their degree of inhibition (Table III). L-3 Methyl histidine was an even more effective inhibitor than L-histidine, while the analogs histamine and imidazole acetic acid caused little or no inhibition. In order to determine if inhibition by the amino acids was additive or cumulative, various combinations were tested, as shown in Table IV. The observed values indicate that inhibition is not additive but more nearly cumulative.

Although L-histidine and L-ornithine were much more inhibitory with Mn^{2+}, they did cause slight inhibition in the Mg^{2+}-dependent assay. The addition of 0.25 mM and 1.25 mM $MnSO_4$ to the Mg^{2+} assay (pH 7.8) did not significantly alter the inhibition caused by L-histidine, L-ornithine, and CAP. However, at pH 6.2 the addition of 0.5 to 1.5 mM $MnSO_4$ (Mg^{2+}, 20 mM) did result in significant inhibition by these compounds (Table V). At this pH, unlike at pH 7.8, L-histidine caused strong stimulation of the Mg^{2+}-dependent activity, if

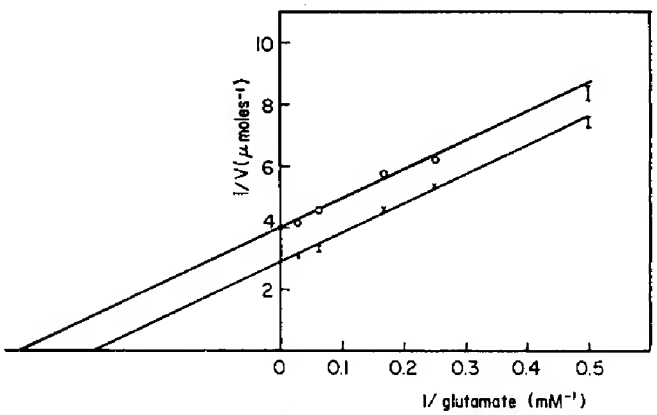

Fig. 7. Inhibition of Mn^{2+}-activated glutamine synthetase by L-serine. The reaction contained 6 mM ATP, 5 mM NH_4Cl, 3 mM $MnSO_4$, and 0.1 M MES-KOH buffer, pH 6.2. Minus serine (×); plus 20 mM L-serine (○). The Pi assay was used (13).

Table IV. *Effect of Combinations of Inhibitors on Mn^{2+}-Dependent Glutamine Synthetase Activity*

The liberation of Pi was assayed. In all cases the reaction contained 3 mM NH_4Cl, 6 mM ATP, 3 mM $MnSO_4$, 0.1 M MES-KOH buffer, pH 6.2, 12 mM L-glutamate, and 0.7 mM mercaptoethanol.

Treatment	Inhibition		
	Observed	Calculated for cumulative	Calculated for additive
mM		%	
A. L-His (20)	63.5		
L-Orn (30)	53.1		
L-His (20) + L-Orn (30)	73.5	83	116.6
B. L-His (4)	19.4		
L-Orn (8)	18.7		
L-His (4) + L-Orn (8)	31.6	34.5	38.1
C. L-Ala (28)	14.8		
Gly (28)	16.7		
L-Ser (28)	16.0		
Ala (28) + Gly (28)	24	28.8	31.5
Ala (28) + Ser (28)	24	28.4	30.8
Gly (28) + Ser (28)	26.6	30	32.7
Ala + Gly + Ser	34.6	37.4	47.5

Table V. *Effect of Addition of Mn^{2+} in Presence of Mg^{2+} on Inhibition Caused by L-Hist, L-Orn, and Carbamyl Phosphate*

In part A, Pi liberation was assayed, and the reaction contained 8 mM ATP, 0.1 M MES-KOH buffer, pH 6.2, 20 mM $MgSO_4$, 10 mM L-glutamate, 4 mM NH_4Cl. In part B, the glutamyl hydroxamate assay was employed, and the reaction differed in that L-glutamate concn. was 30 mM and NH_2OH was 8 mM. The relative activity with histidine, ornithine, and carbamyl phosphate is the activity relative to controls with the same level of $MnSO_4$.

Treatment	$MnSO_4$	Relative Activity
	mM	%
A. Control	0	100
Control	0.5	210
Control	1.5	173
L-His (10)	0	172
L-His (10)	0.5	79
L-His (10)	1.5	62
L-Orn (20)	0	115
L-Orn (20)	0.5	81
L-Orn (20)	1.5	76
B. Control	0	100
Control	0.5	82
Control	1.5	47
Carbamyl phosphate (10)	0	100
Carbamyl phosphate (10)	0.5	54
Carbamyl phosphate (10)	1.5	47

Table VI. *Effect of Several Inhibitors on Mn^{2+}-dependent Glutamyl-Transferase Activity*

The reaction contained 0.5 mM ADP, 20 mM $NaAsO_4$, 8 mM $MnSO_4$, 25 mM L-glutamate, 10 mM NH_2OH, and 0.08 M MES-KOH buffer, pH 6.6.

Treatment	Relative Activity
mM	%
Control	100
L-Glu (25)	92
L-His (12)	106
L-Orn (12)	91
Carbamyl phosphate (12)	54

L-glutamate was limiting and Mn^{2+} was absent. When Mn^{2+} was present at this pH, L-histidine and L-ornithine did inhibit. Also, Mn^{2+} stimulated the enzyme activity at pH 6.2 (unlike at pH 7.8), but only if L-glutamate was limiting (Table V).

Since L-histidine and L-ornithine appear to compete with L-glutamate, it was of interest to test their effect on the activity of the transfer reaction, where glutamine, not glutamate, was the substrate. These results are shown in Table VI, where neither L-glutamate, L-histidine, or L-ornithine had much effect. This does not prove, however, that L-histidine and L-ornithine do bind at the L-glutamate site.

Other Inhibitors. Inorganic phosphate inhibited the enzyme, and was a noncompetitive inhibitor with respect to ATP and L-glutamate (Fig. 8). The *Ki* value for Pi was 40 mM. Pyrophosphate was a more effective inhibitor than Pi, as shown in Figure 9. These results are difficult to interpret, however, for some precipitation of magnesium pyrophosphate occurred, lowering both the true PPi concentration and the Mg^{2+} concentration. Chelation of Mg^{2+} is not the main reason for the PPi inhibition in this experiment, for at 6 mM PPi, even if all of the PPi were complexed to Mg^{2+} there would still be a 6.2 mM excess of free Mg^{2+} present (not complexed to ATP or PPi), which is enough to ensure at least 90% V_{max} (15).

Glucosamine 6-P, which is derived from glutamine, caused only 12% inhibition at 12 mM in (ATP, 6 mM, L-glutamate, 50 mM, NH_2OH, 8 mM, $MgSO_4$, 20 mM). With Mn^{2+}, glucosamine 6-P had no effect. Asparagine and glutamine caused only slight inhibition at concentrations up to 81 mM, while 33 mM aspartate caused 27% inhibition (12 mM L-glutamate was limiting, in these cases).

Carbamyl phosphate caused significant inhibition with Mn^{2+} but much less inhibition with Mg^{2+}, where 5 mM CAP and 10 mM CAP caused only 8% and 22% inhibition, respectively (ATP, 6 mM, $MgSO_4$, 20 mM, L-glutamate, 12 mM, and NH_2OH, 10 mM). The inhibition by CAP was not due to use of the lithium salt, since this cation caused only 8% inhibition

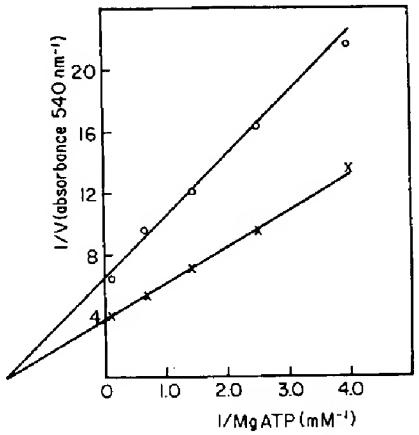

FIG. 8. Inhibition of Mg^{2+}-activated glutamine synthetase by Pi. The reaction contained 4 mM NH_2OH, 60 mM L-glutamate, 45 mM $MgSO_4$, and 0.1 M Tricine-KOH buffer. Minus Pi (×); plus 30 mM Pi (○). Glutamyl hydroxamate synthesis was assayed.

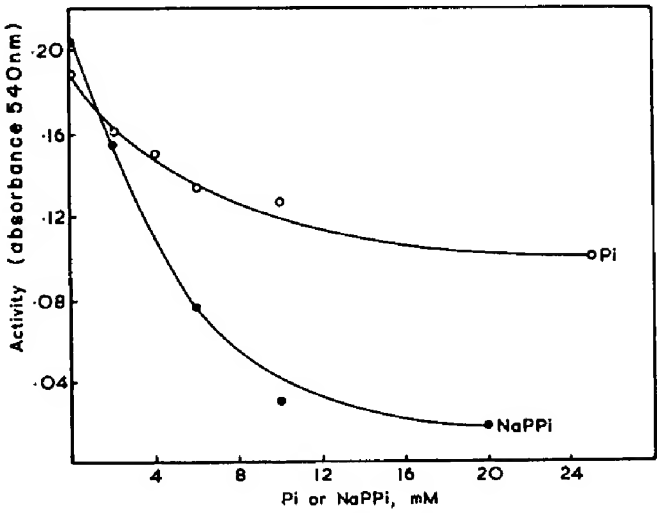

FIG. 9. Effect of Pi and PPi on glutamine synthetase activity. Pi curve: the reaction contained 8 mM ATP, 50 mM L-glutamate, 8 mM NH_2OH, 1 mM diethylenetriamine pentaacetic acid, 40 mM $MgSO_4$, and 0.1 M Tricine-KOH buffer, pH 7.8. PPi curve: as above, but $MgSO_4$ = 20.2 mM. In both cases, glutamyl hydroxamate was assayed.

at 20 mM. Carbamyl phosphate did not appear to be competitive with NH_2OH, ATP, or L-glutamate. In fact, as L-glutamate concentration was increased from 4 mM to 45 mM the inhibition caused by 14.4 mM CAP increased from 40% to 55%, when assayed at pH 5.2 (succinate-NaOH buffer, 0.15 M, pH 5.2, $MnSO_4$, 10 mM, ATP, 8 mM, NH_2OH, 10 mM). Under similar assay conditions increasing $MnSO_4$ from 7 mM to 13 mM or 16 mM increased inhibition by 14.4 mM CAP from 31% to 51%. If assayed at pH 5.2, where Mn^{2+} was always 3 mM in excess of ATP level, then as ATP concentration increased from 0.5 mM to 8 mM the inhibition by CAP rose from 41% to 52%, indicating that ATP must in some manner increase the sensitivity to CAP.

Although it is possible that CAP is not inhibiting directly, but through a product of degradation, the major degradation product, sodium cyanate (24), caused no inhibition at a concentration of 5 mM. Furthermore, the CAP was made up immediately prior to assay, added just before the enzyme, and the assay period was only 5 min, so it is doubtful that much degradation occurred in this time.

DISCUSSION

Of a variety of purine and pyrimidine nucleotides tested, only ADP and 5'-AMP caused significant inhibition of pea leaf glutamine synthetase, and this inhibition was competitive with ATP. While 5'-AMP is inhibitory to glutamine synthetase derived from a variety of other organisms, including *E. coli* (24), *B. Subtilis* (4), *Neurospora crassa* (8), carrot (2), rice (7), and pea seeds (23), apparently the mechanism of this inhibitor varies. With the *E. coli* enzyme, 5'-AMP is a noncompetitive inhibitor with respect to ATP or glutamate, but no data are given with respect to the other sources above. ADP also inhibits the glutamine synthetase from the above sources, and is a more effective inhibitor than AMP. In those cases where it has been studied, it appears to be a competitive inhibitor with respect to ATP (24). It is interesting to note that in the present study 5'-AMP and ADP were much more inhibitory where Mg^{2+} was used than with Mn^{2+}. Since the enzyme is inhibited by ADP and AMP, its activity is modulated by the "energy charge" in a manner similar to many other ATP-utilizing enzymes (1), and the steepness of the slope at higher energy charges is greater with Mg^{2+} than with Mn^{2+} (Fig. 3).

Another product of the reaction, Pi, is also inhibitory, but less so than 5'-AMP. Phosphate inhibition has been reported with the carrot enzyme (2), the pea seed enzyme (23), and the rat liver enzyme (22), although the nature of the inhibition was not reported. The inhibition by PPi in the present study is even greater than that caused by ADP, and it is probably competitive with ATP. Pyrophosphate inhibition of this enzyme from other sources has not been reported to our knowledge.

The lack of a significant degree of inhibition by other purine and pyrimidine nucleotides is unlike the situation existing for glutamine synthetase from many other sources. Glutamine synthetase from most microorganisms is inhibited by CTP or GTP, and these compounds were more inhibitory in the presence of Mn^{2+} than with Mg^{2+} (4, 6, 8). However, these compounds usually had to be present at concentrations of over 2 mM to cause more than 25% inhibition. Glutamine synthetase from carrot is inhibited by CDP, CTP, UDP, UTP, GDP, GTP, using Mg^{2+} (2). Strong noncompetitive inhibition of the rice enzyme by GTP was observed (Mg^{2+} in assay) (7), but since the enzyme was preincubated with GTP (10 min, 35 C) before assay perhaps GTP was simply inactivating the enzyme.

The inhibition observed with alanine, glycine, and serine has been seen with glutamine synthetase from many sources, including *E. coli* (24), rat liver (21, 22), *B. subtilis* (4), and carrot (2). Little or no inhibition by these compounds was seen in the ovine enzyme (22), *N. crassa* (8), or rice (7). This inhibition is usually only weak, but is much greater in the presence of Mn^{2+} than Mg^{2+} (4, 21, 22, 24), as observed in the present study. Tate *et al.* (22) reported that the pea seed enzyme (glutamyl transferase activity) was inhibited 27 to 53% by L-serine, glycine, and L-alanine (Mn^{2+}-dependent activity), where these amino acids were present at 20 mM, which is similar to our results. In most cases, the nature of the binding site for these compounds is not known. In the work described here they do not compete with L-glutamate or NH_4^+. It is interesting to note that β-alanine is a more effective inhibitor than L-alanine in the pea leaf enzyme as well as with the rat liver and ovine brain enzymes (22), but not with *E. coli* (24).

Although arginine synthesis requires glutamine, arginine does not inhibit the pea leaf enzyme, nor those from other sources tested (24). Of other intermediates in the urea cycle, only L-ornithine (Mn^{2+} assay) caused significant inhibition in the present work, and it appeared to compete with L-glutamate. To our knowledge this compound was not tested as an inhibitor with enzymes from other sources.

Carbamyl phosphate requires glutamine for its synthesis, and is required for arginine biosynthesis. This compound did inhibit significantly at sufficiently high concentrations (Mn^{2+}-dependent activity) and was noncompetitive with respect to glutamate, ATP, or NH_2OH. CAP has been found to inhibit glutamine synthetase from most organisms, and usually is much more inhibitory where Mn^{2+} replaces Mg^{2+} (4, 6, 11, 22, 24). It was not tested as an inhibitor with the rice enzyme (7), but 20 mM CAP did cause 50% inhibition with the carrot enzyme (Mg^{2+}-dependent activity) (2). In the case of *E. coli*, CAP was not competitive with any of the substrates (24). In all systems where CAP has been found to be an inhibitor, relatively high amounts (5 to 10 mM) are required for about 50% inhibition. It is significant that in the present study the two compounds (CAP and ornithine required for citrulline and, ultimately, arginine synthesis act as inhibitors.

L-Histidine, also derived in part from glutamine, was among the most effective inhibitors tested in the present work. As with all other inhibitors except ADP and AMP, histidine was much more inhibitory in the presence of Mn^{2+} than with Mg^{2+}. This is again similar to the case for the enzyme from other organisms (4, 6, 8, 22, 24), although the pea leaf enzyme appears to be more sensitive to histidine inhibition than those of other sources. The rat liver enzyme was inhibited by histidine only in the presence of Mn^{2+}, and histidine was competitive with L-glutamate (22). The rice enzyme (Mg^{2+}-dependent activity) was not inhibited by 20 mM histidine, (7), while the carrot enzyme (Mg^{2+}-dependent activity) was actually stimulated 15% by 30 mM histidine (2). The fact that inhibition by histidine did not occur where the glutamyl transfer assay was used suggests that it does not compete with glutamine, as opposed to glutamate. This was analogous to the situation observed with ornithine, another glutamate competitor.

It should be emphasized that the "competitive inhibition" caused by histidine and ornithine does not in itself prove they are truly competing with L-glutamate for the glutamate binding site—alternative explanations are possible.

At this point it is difficult to interpret the *in vivo* significance, if any, of the inhibition caused by glycine, alanine, serine, CAP, ornithine, and histidine. First, only with Mn^{2+} do they inhibit significantly and whether Mn^{2+} (or MnATP) ever replaces Mg^{2+} (or MgATP) in this reaction in plant cells is unknown and is perhaps unlikely owing to the much greater *in vivo* concentrations of Mg^{2+} than Mn^{2+} (12). Second, the concentrations

of the above inhibitors needed for 50% inhibition are high: 1.7 to 6.5 mM for histidine, 10 to 12 mM for CAP, 7.8 mM for ornithine, and over 40 mM for glycine, serine, and alanine. It can be questioned whether these intermediates ever accumulate in such amounts. The cumulative nature of these inhibitors could result in significant inhibition where most or all of them were present simultaneously in more moderate amounts. Actually, with the glutamine synthetase of most organisms all of the individual inhibitors mentioned previously must be present in millimolar concentrations for significant inhibition to occur, and it is not yet clear whether such regulation occurs *in vivo*. With respect to the present work, it should be pointed out that even though ornithine and histidine must be present at levels of about 2 to 8 mM for 50% inhibition, their K*i* values are about equal to (for ornithine) or $\leq$ half (for histidine) the K*m* for glutamate, so that if the histidine and ornithine levels *in vivo* are only half the glutamate level, significant inhibition may occur.

It is more likely that pea leaf glutamine synthetase activity is regulated by the energy charge of the cell. With Mg^{2+}, the relative activity at an energy charge of 0.8 and 0.5 were 53% and 20%, respectively, while with Mn^{2+} the relative activities were 73% and 49%, respectively. It appears that a significant amount of glutamine synthetase is present in the chloroplasts (14) where the energy charge in the light ranges from 0.66 to 0.76 in isolated illuminated spinach chloroplasts but only 0.50 to 0.58 in isolated nonilluminated chloroplasts (10, 17). Recently, Canvin and Atkins (3) showed that barley and bean leaves incorporated ammonia ($^{15}NH_4^+$) into amino acids about five times faster in the light than in the dark, an observation which supports the effect of energy change on glutamine synthetase.

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